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### ERRATA

- Vol. 19, page 37, line 4, "from 10 to 15 per cent" should read "from 1.0 to 1.5 per cent"
- Vol. 19, page 87, line 12 in SUMMARY, should read "percentage dry weight of leaf . . . of alcohol-soluble. . . ."
- Vol. 19, page 100, Table II, line 4 in columns headed Molar Ratios, "1" should read "2"
- Vol. 19, page 102, line 4 should read "The greater percentage dry weight. . . ."
- Vol. 19, page 303, next to last sentence in first paragraph of SUMMARY, mean spore volume for *Cephalosporium acremonium* should read "5.11"
- Vol. 19, page 305, line 21, "var. Bountiful" should read "var. Pinto" and "Pinto variety" should read "Bountiful variety"
- Vol. 19, page 309, line 5 from bottom, "500  $\mu$ " should read "500  $\mu^3$ "
- Vol. 19, page 417, next to last line of SUMMARY, "homo" should read "homo-"
- Vol. 19, page 437, line 4 in title should read "QUANTITIES"
- Vol. 20, page 43, photograph belongs on page 53.
- Vol. 20, page 53, photograph belongs on page 43.

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Vol. 20, page 89, Table III, headings for columns 2 and 3, and columns 6 and 7, should read "Meq./leaf  $\times 10^4$ "

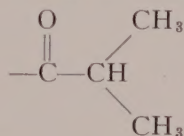
Vol. 20, pages 115, 116, 118, legend for X axis of Figures 1, 2, and 3 should read " $R_f \times 10$ "

Vol. 20, page 121, line 2 from bottom should read "Contract AT(30-1)-2413"

Vol. 20, page 123, running title, "Weinsten" should read "Weinstein"

Vol. 20, page 126, legend for Y axis on right side of Figure 3 should read " $C^{14}O_2$  EVOLVED C.P.M.  $\times 10^{-3}$ "

Vol. 20, page 155, Table I, column 3, last formula should read



Vol. 20, pages 158, 159, 160, legend for Y axis of Figures 1, 2, and 3 should read "UPTAKE- $\mu\text{G.}/\text{G.} \dots \times 10^{-3}$ "

Vol. 20, page 218, Table I, column 4, line 11 should read "III = IV"

Vol. 20, page 242, last line, "2 per cent" should read "0.2 per cent"

Vol. 20, page 244, Figure 3, legend, "Dowex 1-X, 10 resin" should read "Dowex 1-X<sub>10</sub> resin"

Vol. 20, page 272, line 11, "growth. *N.* . . ." should read "growth, *N.* . . ."

Vol. 20, page 281, line 6, "areas (20); of . . ." should read "areas (20); or . . ."









PERCY W. ZIMMERMAN

1884-1958

## PERCY W. ZIMMERMAN

(February 23, 1884—August 14, 1958)

A. E. HITCHCOCK

When Dr. P. W. Zimmerman succumbed on August 14, 1958, at Wenatchee, Washington, the world lost a well-known scientist and a beloved man. It is rarely that one sees a scientist with such broad interests and deep understanding of human nature. His inquiring mind led him into many areas of plant life on and off the beaten pathways. In this never-ending search for additional knowledge about plants he made many friends and acquaintances in all walks of life.

Apart from his scientific work he devoted much time to community services and was always keenly interested in the personal affairs of the people around him. By mail and in person many individuals kept him posted as to their progress and family status. In such an active busy life his only concern was the possibility that physical debilitation would inactivate him before life had run its course. He was spared this fate when an embolism caused his death following an emergency operation.

"Zim," as nearly all his close associates affectionately knew him, was born in Manito, Illinois, on February 23, 1884. He taught in Illinois schools and became Superintendent of Schools (1910 to 1913) in Westville, Illinois. His natural interest in biology was developed at an early age on the farm and was greatly increased as a student at the Eastern Illinois State Normal School at Charleston, where he became associated with the late Otis W. Caldwell, at that time Head of the Department of Biology. Dr. Caldwell moved to Chicago in 1907 and became Dean of the University School of Education in 1913. Dr. Zimmerman followed his former teacher to Chicago in 1914 and became his assistant in Botany in 1916. The following degrees were granted to him by the University of Chicago: B.S. 1915, M.S. 1916 and Ph.D. 1925. He went to Maryland State College (merged with the University of Maryland in 1920) as Associate Professor of Botany in 1916. He was appointed Acting Dean in 1917 and Dean of the College of Agriculture from 1918 to 1925, after which he became associated with Boyce Thompson Institute for Plant Research, Inc., in Yonkers, New York.

In scientific circles throughout the world Dr. Zimmerman was known for his investigations relating to vegetative propagation, plant hormones and growth regulants and the effects of gases and air pollutants on plants. He received many honors and awards, among which were the following shared with his associate: in 1932 the A. Cressy Morrison Award of The New York Academy of Sciences for work relating to the initiation and stimulation of roots from exposure of plants to carbon monoxide gas (the



first use of a known chemical for inducing the formation of a plant organ), in 1935 the Annual Award of the American Association for the Advancement of Science for pioneering work in the field of plant hormones and growth regulants (the first A.A.A.S. award to a botanist) and in 1946 the Vaughan Award of the American Society for Horticultural Science for the results obtained with substituted phenoxy acids and other growth regulants that were effective for increasing fruit set and inducing the formation of seedless fruit.

Dr. Zimmerman's interests in factors affecting the growth and development of plants were remarkably broad in scope. He was author and co-author of 231 publications relating to rooting of cuttings, effects of gases and air pollutants on plants, the response of plants to natural and synthetic growth regulants and the relation between chemical structure and physiological activity. To laymen and scientists alike he was known especially for his work on the selection, propagation and growth of winter hardy strains of holly and camellia.

He was responsible for the large-scale growing of hardy strains of American (*Ilex opaca* Ait.) and English (*Ilex aquifolium* L.) holly in the vicinity of Yonkers, New York, and northward. A collection of 5,000 holly plants grown from seed and from cuttings was set out in a trial plot five years prior to the subzero temperatures ( $-18^{\circ}$  to  $-25^{\circ}$  F.) during the winter of 1933 to 1934. From this collection hardy strains of holly with desirable leaf, fruit and growth characteristics were released to the trade. Similarly, he selected, propagated and grew strains of *Camellia* which have proved winter resistant in the vicinity of Yonkers for various periods up to 30 years. His interest in *Camellia* started as a hobby about 1927 when he received cutting material of unknown parentage from the State of Washington. Plants grown from these cuttings have been referred to in published reports as the "Z" strain of *Camellia japonica* L.

Many of the growth regulants used in practice were first tested in Dr. Zimmerman's laboratory. These included  $\beta$ -indolebutyric and  $\alpha$ -naphthaleneacetic acids, several substituted phenoxy acids including 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and chlorosubstituted benzoic acids. Their practical uses include rooting of cuttings, preventing the preharvest drop of apples and other fruit, increasing fruit set and inducing seedless fruit formation, thinning of fruit, regulating the flowering of pineapple, increasing the size of and hastening the ripening of fruit and killing or inhibiting the growth of weeds.

From the early testing of 2,4-D in Dr. Zimmerman's laboratory to the large-scale use of this chemical as an herbicide represents one of the outstanding developments in the history of agricultural chemicals, equaling or perhaps surpassing the use of DDT as an insecticide. Although 2,4-D is probably best known for its selective action in controlling weeds in crops,



especially cereals, it has been widely used, together with 2,4,5-T, for killing brush and other types of vegetation, including poison ivy. The remarkable effectiveness of 2,4-D for killing water hyacinths in Louisiana was demonstrated by experimental work carried out over a period of two years (1948 to 1950) under the supervision of Dr. Zimmerman. In one instance helicopter-applied sprays of 2,4-D caused complete killing of all water hyacinths in an isolated waterway belonging to a hunting and fishing club near Norco, Louisiana. Since no water hyacinths have appeared in the treated area to date (10 years), the results are unique in demonstrating large-scale eradication of a single species in a specific area.

On the basis of the results with water hyacinths in Louisiana, Dr. Zimmerman was asked to arrange for a similar eradication program in the Belgian Congo, Africa. The recommendations and subsequent inspection of results made by a former assistant indicate that successful control of water hyacinths in the tributaries of the Congo River has been accomplished.

Dr. Zimmerman was a pioneer in studying the effects of air pollutants on plants. The work with ethylene, other unsaturated hydrocarbon gases and sulfur dioxide started in the late 1920s. Since 1951 special emphasis has been placed on the effects of fluorides on plants. It was his foresight and concerted efforts which made possible the Fluoride Project now in its ninth year at Boyce Thompson Institute. Thus it is understandable why he was asked to organize the Agriculture Panel for the first United States Technical Conference on Air Pollution held in Washington, D. C., May 3-5, 1950. Former President Harry S. Truman requested that a Federal Interdepartmental Committee be formed to sponsor this particular air pollution conference which was composed of six other panels besides the one on agriculture. Later, Dr. Zimmerman took an active part in air pollution programs in various parts of the country, and he was on a field trip to investigate the possible effects of air pollution on plants in Wenatchee, Washington, just before his death.

His method of experimentation and reporting of results was unique. He did not think in terms of a balanced experimental design or statistical interpretation of results. This led to experimental procedures involving many tests that were repeated under different conditions. Results were then selected which he thought best illustrated how the test plants responded to various treatments. Precise quantitative results were thus sacrificed for more extensive qualitative and semi-quantitative data, much of which was not published.

These additional unpublished data and unrecorded observations aided in the formulation of concepts and conclusions that were better founded than the reader realized. It is to Dr. Zimmerman's credit that no important concept or conclusion of his has been refuted to date. He rejected the idea of Auxin A and B from its inception and lived to see it buried as a scientific

fact. He was one of the few who opposed the generally accepted concept that indoleacetic acid is the primary growth regulant (auxin) physiologically active in normal growth. He lived to see this concept refuted. He expressed grave doubt as to the validity of superficial ideas regarding the relationship of chemical structure to growth regulant activity in the aryl-oxy acids and saw his doubts verified as one hypothesis replaced another in rapid succession.

It could not be said that Dr. Zimmerman typified the modern scientist. Although he saw the era of specialization ushered in, his broad and varied interests prevented him from following a highly specialized pathway in science as did most of his colleagues. He was botanist, plant physiologist, horticulturist, farmer, gardener, beekeeper and a confidential counsellor to many of his friends and acquaintances. His work was his hobby and any of the time he spent working with plants was vacation time to him. He made sure that there was always an end-product from experimental work and hobby activities which could be given to his friends and associates. No one could understand how he could give so much time and energy to friends and acquaintances and to outside activities and still accomplish the many things for which he was well known professionally.

The amazingly broad scope of Dr. Zimmerman's activities may be judged by the many affiliations he had with various scientific groups such as: American Society for Horticultural Science; Botanical Society of America; Torrey Botanical Club (President 1946); American Association for the Advancement of Science; American Chemical Society; Westchester Chemical Society; Weed Society of America; Florida State Horticultural Society; American Horticultural Council; Camellia Society of the Potomac Valley; American Institute of Biological Sciences; Air Pollution Control Association; Advisory Committee on Air Pollution, Interstate Sanitation Commission; Board of Managers, The New York Botanical Garden; Sigma Xi; Alpha Zeta; Phi Kappa Phi; Pi Alpha Xi.

His interests in community service were equally broad since he served as: President, Board of Managers, Yonkers Bureau of Laboratories; First Vice Chairman, Board of Trustees, St. John's Riverside Hospital; District Director of Boy Scouts; Director of Home Service, Yonkers Chapter, American Red Cross during World War II; Chairman, Committee on Child Care, Development and Protection of the Yonkers War Council during World War II; Member Family Service Society; Member Board of Directors, Salvation Army; Member Rotary Club, Yonkers; Member Hudson River Country Club; Honorary Member, International Mark Twain Society.

Throughout his life Dr. Zimmerman never ceased being a teacher. This helps to explain why he wished always to be the center of attention in any group and why in committee meetings his services were outstanding when

he was chairman. Dr. Zimmerman gave many talks to various groups, locally and throughout the country, about his own and other work at Boyce Thompson Institute. In fact, he served as a good will ambassador for the Institute to the point where the man and the institution were synonymous to some people.

Dr. Zimmerman was known as an interesting rather than a good speaker, and he used live material frequently, as well as slides, to supplement his talks. His innate confidence, tinged with a bit of conceit, led him to believe that each talk was the best he had ever given. When confronted with the unrealistic relativity of such a claim, he had no comment to make. However, in spite of a few such idiosyncrasies, he will be remembered as a kindly man of many talents who gave much of his time and energy to his fellow men, laymen and scientists alike.

The passing of Dr. Zimmerman affected many people in all walks of life and in all parts of the world. There will never be another man like him. Thus he becomes legendary to future generations.

Dr. Zimmerman is survived by his wife, the former Patti C. Martin, whom he married on August 16, 1911; a daughter, Mrs. William (Lois Josephine) Gerow of Elm Grove, Wis.; two sons, Jack Martin Zimmerman of Houston, Texas, and Robert Louis Zimmerman of Aiken, S. C.; and a grandson, James Zimmerman of Houston.





# OBSERVATIONS ON THE MOVEMENT OF INJECTED DYES IN *PINUS PONDEROSA* AND *ABIES CONCOLOR*

J. P. VITÉ<sup>1</sup>

## SUMMARY

Acid Fuchsin and other dyes in aqueous or alcoholic solution were introduced into the water-conducting layers of *Pinus ponderosa* Laws. and *Abies concolor* (Gord. & Glend.) Lindl. by different methods of trunk injection. The trees were then cut into logs, and disks were removed at different heights to determine the pathway taken by the dye. The dye was found to ascend along tracheidal conduits that follow the slope of grain in the sapwood. The structure in which these conduits are arranged is basically the same in both tree species. The conduits deviated to the left of the tree axis and changed continuously to the opposite side in each subsequent growth ring. Accordingly, dye injected radially through a hole bored in the lower bole produced a typical spiral pattern as observed in transverse sections in the upper bole. This also resulted in supplying most branches of the tree with dye in an inner or outer growth ring or in several growth rings. Besides continuous changes in the deviation of the tracheidal conduits according to age, sudden changes in angle and direction were produced by varying environmental conditions involving the width of the annual growth rings. Heavy pruning and releasing suppressed trees were found to have similar effects.

The results of dye injections confirmed the recent concept of several wood technologists that a varying slope of grain is the rule rather than the exception in conifers. Furthermore, the experiments indicated an arrangement of the tracheidal conduits which led to the conclusion that in the tree species investigated not only the transport but also the distribution of the transpiration stream over the whole transpiration surface is accomplished by the sapwood.

## INTRODUCTION

Experiments in which dyes are introduced into woody plants and their translocation with the ascent of sap is observed are among the older investigations in botany. In the nineteenth century, perhaps the most frequent attempt was to incorporate dyes into standing trees for wood coloration. However, the reception of suitable dyes by the trees was incomplete and only a few references are known on this subject. Since the late nineteenth century the literature on plant protection and applied botany contains numerous articles on the injection of trees with dyes. Most of these experiments were limited to hardwoods, principally fruit and ornamental trees. Forest trees, especially conifers, have not been studied extensively. The progressive development and use of systemic insecticides as well as the growing use of other chemicals in a similar way for debarking (10), chemotherapy (19, 20, 29, 39, 43), bark beetle control (3, 7, 21) and wood preservation in standing trees (18, 34) demand a better understanding of the transpiration stream.

<sup>1</sup> Boyce Thompson Institute, Forest Research Laboratory, Grass Valley, California. Copyright, 1959, by Boyce Thompson Institute for Plant Research, Inc.

The first complete communication on the movement of dyes in living or dead plant tissues or capillary spaces was written by Goppelsroeder (11) in 1901. A compilation of early work, dealing mainly with fruit trees but including early experiments on conifers, is to be found in a monograph by Müller (29) on plant injection. Baker and James (1) studied the behavior of dyes in the transpiration stream of sycamores. More recently, Roach (37) has published several papers on plant injection and injection procedures. The material used varied from strawberry plants to fully grown fruit trees. He also gives a complete history of the injection work done earlier throughout the world.

Dye injections were used by Greenidge (12, 13, 14) to study the movement of moisture, especially the lateral transfer, in large woody stems. He employed methods causing drastic disruption of the normal channels of water transport similar to those of Elazari-Volcani (8) and Preston (36). Prior to incision of the entire circumference of the bole or to the partial injection, the trunks of trees (mainly deciduous trees) were sawed more than half through at one or two overlapping levels.

The author's investigations originated from attempts to control forest insects by tree-trunk applications of systemic insecticides. Originally, the trunk-wrapping method developed by Jeppson *et al.* (20) and used by Jancke (19) was employed (40). Later, success with trunk injections consisting of single shots into the lower bole brought up the question of the causes of the complete distribution of the injected material over the tree. Experiments with dyes showed that, in the tree species under consideration, the answer lay in the particular structure of the tracheidal conduits (30, 41, 42).

Again, the same method was applied and further developed when the transpiration stream of ponderosa pine became the subject of an investigation of the relationship between water supply in this conifer and bark beetle attacks. Besides the literature mentioned above these investigations followed two other sources: the earlier research on the transpiration stream of conifers (24, 25, 26, 38) together with the recent discussions of water conduction in plants (2, 6, 15, 16, 17, 23); and the research on the slope of grain in the wood of conifers which has been a problem of wood technology for almost a century (4, 5, 9, 27, 28, 31, 32, 33, 35).

The present paper describes methods employed and the results obtained in a study of the ascent of dye in the transpiration stream of ponderosa pine with some additional observations on white fir. The water-conducting systems of both species, as indicated by dye patterns, show the same general structure which differs from certain other conifers (45). These findings will be discussed in relation to the technological problem of the slope of grain in conifers (44) and in relation to the ecology and phylogensis of some groups of conifers.

## MATERIALS AND METHODS

Dye was introduced into the water-conducting layers of the sapwood of some 70 specimens of *Pinus ponderosa* Laws.<sup>2</sup> from 4 to about 95 years of age and 9 trees of *Abies concolor* (Gord. & Glend.) Lindl. between 60 and 100 years old. The experiments were carried out between May 7, 1957 and August 15, 1958 in the Boyce Thompson Institute Experimental Forest at Grass Valley, the Zeibright Mine tract, New Verde Mines Company, and the Tahoe National Forest, Bloomfield ranger district, Nevada County, California.

## INJECTION TOOLS AND PROCEDURES

During the experimentation the following procedures were employed:

(a) *Injection into a bored hole.* Conifers, especially those with high oleoresin content, were treated by means of a hole, 0.5 to 2.0 cm. in diameter, which was bored radially through the sapwood and closed by a cork stopper. A tube through the stopper supplied the hole with the dye solution from a bottle, burette or test tube (Fig. 1).

(b) *Knife injection.* The "knives" employed for injection are small metal cylinders with a one-inch-long hollow blade for driving into the sapwood. Two openings near the tip of the blade provide for the transmission of the dye solution into the trunk (42). The split in the bark at the site of injection was sealed with metal and rubber plates and putty.

(c) *Incision.* In the laboratory, the base of the tree was simply placed in the dye solution. In the field, incisions were made through the whole sapwood, leaving the tree standing and the heartwood uncut. An inverted cone frustum fitted to the bole supplied the cut with liquid. It is not necessary to perform these incisions in conifers under water (43) or under the surface of the injection liquid as suggested for other trees (2, 12).

(d) *Root injection.* In the field one root was excavated, cut off and immersed in the dye solution. Similarly, the severely pruned roots of trees treated in the laboratory were set in water, but one longer side root was immersed in the dye solution.

## DYES AND DYE SOLUTIONS

Following a recommendation of Roach (37), Acid Fuchsin was generally used in concentrations of 0.3 to 1.0 per cent. Of the various dyes previously recommended, only eosin proved useful. It ascends more slowly than Acid Fuchsin, and concentrations adequate for the purpose (0.6 to 2.0 per cent) are phytotoxic to cambium and needles. As a very slow traveling dye, Acridine Red was sometimes used for triple injections to locate the tracheidal conduits in three different spots on the same trunk level.

<sup>2</sup> Scientific and common names used herein follow Little (22).



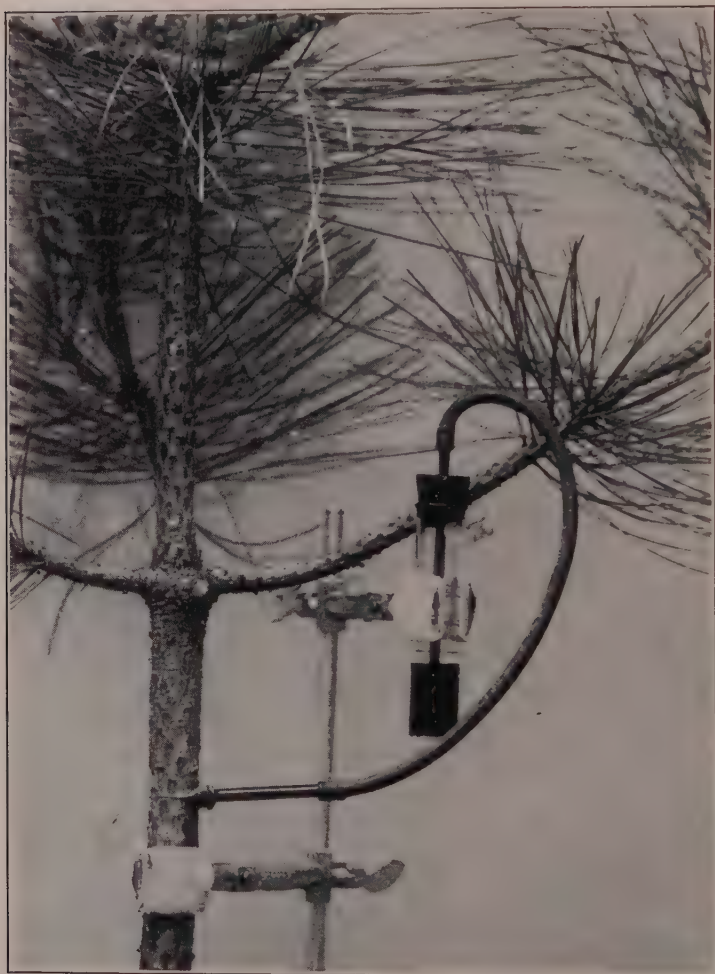


FIGURE 1. Injection procedure by means of a hole bored into the trunk. A test tube supplies Acid Fuchsin dye.

#### RATE OF UPTAKE AND ASCENT OF THE DYES

The absolute quantity of dye solution taken in by a standing tree depended upon the size of the injection hole and the rate of transpiration of the tree at the time. The rate of uptake from each injection varied from 40 to 180 ml. per day in a smaller tree to 450 ml. of aqueous solution in larger trees. Occasionally, unusually high absorption was observed for a limited time. During an afternoon thunderstorm with heavy rain, a ponderosa pine absorbed through each of three injection holes between 40 and 62 ml./hr. In general, the uptake of injected liquids followed the diurnal variation of

the diffusion pressure deficit and occurred faster during the afternoon hours or after the noon depression of transpiration.

Dye injected into the sapwood of a tree ascended only as fast as the cell walls of the conducting elements adsorbed it. The more rapid rate of movement of Acid Fuchsin was evident. In aqueous solution it traveled on the average about four times as fast as eosin or Acridine Red which seemed to be even more limited in transport. The alcoholic solutions were always translocated much faster than the aqueous solutions, in spite of the dilution of the former with the moisture of the sap (Table I).

In trees with heavy oleoresin discharge the alcoholic solutions were also

TABLE I  
RATE OF ASCENT OF VARIOUS DYE SOLUTIONS INJECTED INTO THE XYLEM OF  
PONDEROSA PINE UNDER FIELD CONDITIONS  
(JUNE 12-14, 1957)

| Type of solution                            |            | Time       | Xylem,<br>temp. ° C. | Ascent<br>in cm. | Rate of<br>ascent,<br>cm./hr. |
|---------------------------------------------|------------|------------|----------------------|------------------|-------------------------------|
| Ingredients                                 | Concn. (%) |            |                      |                  |                               |
| Acid Fuchsin<br>H <sub>2</sub> O            | 0.8        | 1:30 P.M.  | —                    | 0                | —                             |
|                                             | 99.2       | 3:15 P.M.  | —                    | 15               | 8.6                           |
|                                             |            | 7:20 A.M.  | 13                   | 120              | 7.5                           |
|                                             |            | 10:30 A.M. | 20.2                 | 32               | 10.5                          |
|                                             |            | 3:30 P.M.  | 24.2                 | 177              | 35.4                          |
| Acid Fuchsin<br>H <sub>2</sub> O<br>Alcohol | 0.8        | 1:45 P.M.  | —                    | 0                | —                             |
|                                             | 49.6       | 8:45 A.M.  | 13.1                 | 177              | 9.3                           |
|                                             | 49.6       | 12:30 P.M. | 16.1                 | 118.5            | 31.2                          |
|                                             |            | 3:00 P.M.  | 18.4                 | 156.5            | 62.6                          |
| Eosin<br>H <sub>2</sub> O                   | 1.6        | 1:30 P.M.  | —                    | 0                | —                             |
|                                             | 98.4       | 3:15 P.M.  | —                    | 7                | 4.0                           |
|                                             |            | 7:20 A.M.  | 13                   | 25.5             | 1.6                           |
|                                             |            | 10:30 A.M. | 20.2                 | 3.5              | 1.2                           |
|                                             |            | 3:30 P.M.  | 24.2                 | 19.0             | 3.8                           |
| Eosin<br>H <sub>2</sub> O<br>Alcohol        | 1.6        | 1:45 P.M.  | —                    | 0                | —                             |
|                                             | 49.2       | 8:45 A.M.  | 13.1                 | 143.5            | 7.6                           |
|                                             | 49.2       | 12:30 P.M. | 16.1                 | 47               | 12.5                          |
|                                             |            | 3:00 P.M.  | 18.4                 | 15               | 6.0                           |
| Acridine Red<br>H <sub>2</sub> O            | 1.6        | 2:30 P.M.  | —                    | 0                | —                             |
|                                             | 98.4       | 8:45 A.M.  | 13.1                 | 30               | 1.7                           |
|                                             |            | 12:30 P.M. | 16.0                 | 0                | 0                             |
|                                             |            | 3:00 P.M.  | *                    | 0*               | 0                             |
|                                             |            | 7:30 A.M.  |                      | 0*               | 0                             |
|                                             |            | 10:30 A.M. |                      | 0*               | 0                             |
| Acridine Red<br>H <sub>2</sub> O<br>Alcohol | 1.6        | 1:45 P.M.  | —                    | 0                | —                             |
|                                             | 49.2       | 8:45 A.M.  | 13.1                 | 123.5            | 6.5                           |
|                                             | 49.2       | 12:30 P.M. | 16.1                 | 20.5             | 5.5                           |
|                                             |            | 3:00 P.M.  | 18.4                 | 10               | 3.9                           |
|                                             |            | 7:50 A.M.  | 13.0                 | 59               | 3.5                           |
|                                             |            | 10:45 A.M. | 20.2                 | 4                | 1.3                           |

\* Constant, but gaining intensity in color.

taken up much better than aqueous liquids. To determine a suitable concentration, different alcoholic solutions were tested by means of blotting paper. The results of these tests indicated the rate of ascent of alcoholic solution to be linear with respect to concentration. After 12 hours, 97 per cent alcoholic solution traveled 12 cm. and the rate decreased 2.4 cm. for each 10 per cent decrease in ethanol content. Below 20 per cent ethanol the tractive forces were essentially the same as for distilled water (34 cm./12 hours). The rate of dilution varied widely in different trees as well as with the distance from the point of injection. However, adequate results were obtained by using higher concentrations of alcohol (up to 50 per cent) for larger trees and lower concentrations (10 to 20 per cent) for smaller trees. About 0.6 to 1.0 per cent dye was used for trees taller than 40 feet and 0.3 to 0.4 per cent for small trees.

#### EVALUATION

Following injection the tree was sectioned, and the stained pattern traced from transverse sections. Before sample disks were removed, the parallel to the tree axis through the injection hole ("axis-parallel") was projected, and the heights of the disks above injection were marked by means of nails and tags to preserve the original relation of the samples in the tree. Other figures recorded were the height of injection above the ground, the age and height of the tree, and the length and shape of the active crown.

In the laboratory, the surface to be observed was cut with a bandsaw immediately before the stained pattern was traced on transparent paper. In addition, the tracing included the outermost growth ring of the xylem, the pith and the crossings of the stained pattern through certain growth rings. As a matter of custom, the growth rings were counted from the outside towards the center and designated by their year. The chosen growth rings were also marked on the sample disks.

Further evaluation consisted of measuring the distance from the junction of a selected growth ring with the stained pattern to the axis-parallel that ran vertically through the injection point. The distance was measured along the summer-wood of the growth ring under observation. This distance gave the relative deviation of the tracheidal conduits from a parallel run to the tree axis. This rather simple evaluation was satisfactory when only the general structure of the xylem or the water-conducting system was studied. It showed the actual relation of the tracheidal conduits of different growth rings to each other on the same level, but did not give the true angles of deviation. To obtain approximate values for the true deviation of the conduits or the slope of grain along the tree, some additional data and procedures had to be employed.

Exact evaluation of the true deviation is difficult because of the ir-

regular form of a stem. The shape of the stem can be most closely represented by a family of concentric cone frusta formed by each succeeding growth ring. Theoretically, one can conceive of the layer of a growth ring unfolded into a plane, the base of which coincides with the level of the injection. In a two-dimensional display of the cone frustum the circumference of the tree would be represented by an arc of large radius. It can be shown that the difference between the length of the arc and of the subtended

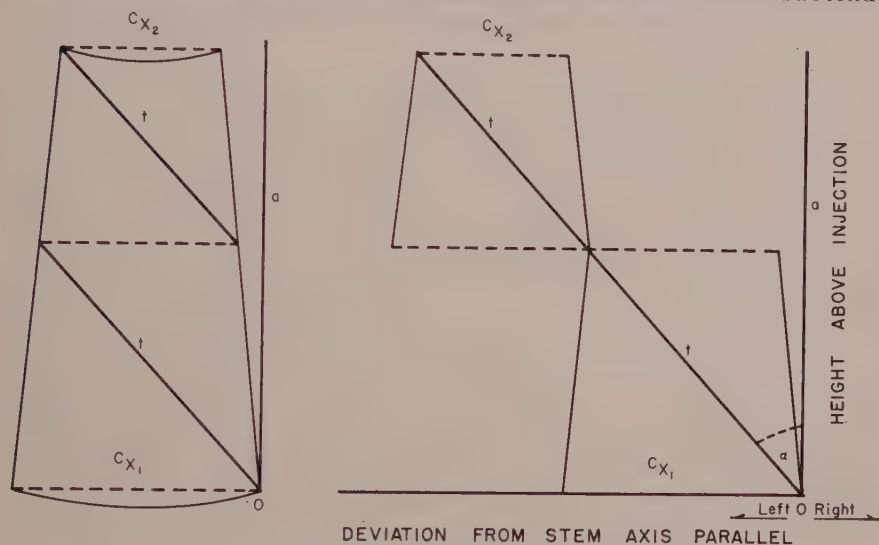


FIGURE 2. The plane of a cone frustum representing the unfolded layer of a growth ring.  $c_{x1}$  Basal cord,  $c_{x2}$  apical cord of the growth ring layer in which the tracheidal conduits ( $t$ ) ascend in a helix of 2 revolutions.  $a$  = Parallel line to the tree axis running through the point of injection ( $O$ ). Right: the tracheidal conduits are represented in continuous deviation from the stem axis parallel ( $a$ );  $\alpha$  = angle of deviation. Further explanation in text.

chord is negligible and thus the calculations are based on the chords representing the measured circumferences for simplicity. Perpendicular to the basal chord is the parallel to the stem axis forming thus a system of rectilinear coordinate axes. The abscissa in Figure 2 shows the deviation ( $x$ ) of the dyed tracheidal conduits ( $t$ ) from the stem-axis parallel ( $a$ ), and the ordinate shows the position of the transverse sections above injection ( $O$ ). This holds true only until the tracheidal conduits leave the plane of the layer, i.e., until the first revolution is completed. After the first revolution, the conduits appear on the same level but on the opposite side of the plane (Fig. 2). Accordingly, one can compute the approximate deviation at any particular point according to the formula

$$x = \frac{c_{x1} + c_{x2}}{2} m,$$



where  $c_{x1}$  and  $c_{x2}$  represent the circumferences at the extremes of a given trunk section; and  $m$  is the number of revolutions of the tracheidal conduits around the stem axis between  $c_{x1}$  and  $c_{x2}$  level. In order to present the entire run of a tracheidal conduit graphically, one can divide the plane of a growth ring into sections of completed revolutions and connect these sections diagonally (Figs. 2, 3). The approximate, true angle of deviation, which is conventionally measured as the deviation from the axis parallel, can be obtained from the same figure as  $\tan \alpha = x/a$  according to the tangent formula.

An example will illustrate the application of this formula (Fig. 3). A 97-year-old white fir was injected, sectioned by 8 transverse cuts between 84 and 1026 centimeters above injection level, and the helical run of the tracheidal conduits of the growth rings of 1926, 1938 and 1957 were traced. This was first done by plotting the readings directly into the coordinate system (dotted lines). These points represent the relative degree of deviation between tracheidal conduits of various growth rings within a given transverse section. For instance, the tracheidal conduits of the growth ring 1957 complete in 1026 cm. above injection level a helix of  $1\frac{1}{8}$  revolutions. In this transverse section, the circumference of the growth ring 1957 was 32 cm. and the apparent deviation was 34 cm. Since in the lower bole the helix followed a much larger circumference, the actual deviation of these tracheidal conduits between any two transverse sections may be closely approximated by the use of the formula mentioned above. Considering the circumference of the injection level of 61 cm. the deviation in the 1026-cm. section was

$$\frac{61 + 32}{2} \times 1\frac{1}{8} = 49.4 \text{ cm.}$$

The solid lines in Figure 3 compare the run of the tracheidal conduits obtained by this procedure with the directly transferred, apparent deviations.

## RESULTS AND DISCUSSION

Injections through a bored hole or an injection knife proved especially suitable. Compared with these procedures, neither incision nor the root injection was particularly helpful for an investigation on the movement of dyes. In agreement with Greenidge (12), it was found that only small amounts of dye solution were absorbed from root injections, quantities usually inadequate to supply the whole tree. Incision procedures resulted in widespread stained patterns which did not allow a discriminative evaluation of differences in various layers of the sapwood. Only the hole and knife injections gave a clear picture of the axial ways of transport which, in the case of conifers, consist of vertically adjoining tracheids providing conducting channels or conduits. In ponderosa pine and white fir, the dye

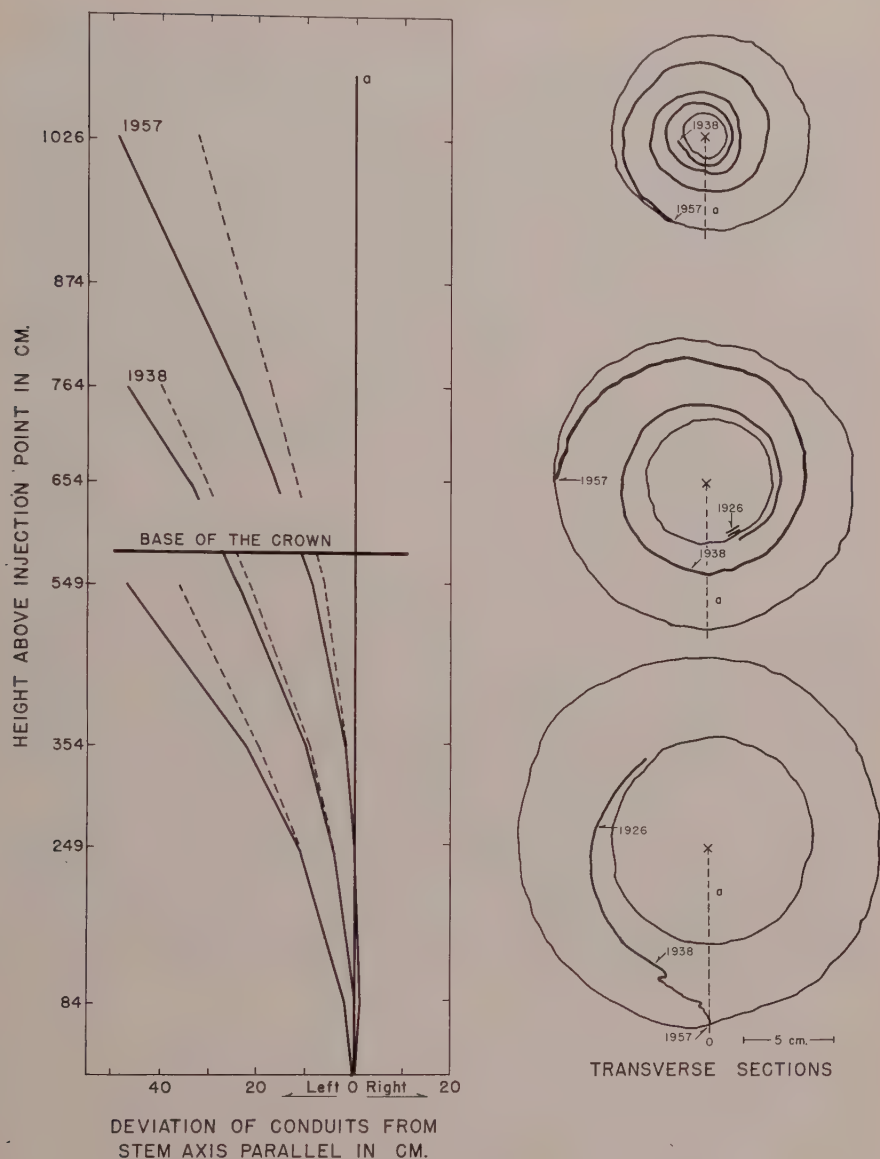


FIGURE 3. Transverse sections through the bole of a 97-year-old white fir at 249, 654 and 1026 cm. above the point of injection (O) and the graphic presentation of the run of the tracheidal conduits in the growth rings of 1926, 1938 and 1957 (solid lines); the dotted lines represent the apparent run of the tracheidal conduits as explained in the text. The abscissa shows the deviation in centimeters of the conduits from the stem axis-parallel (a), the ordinate the distances above injection in centimeters.

injections always resulted in very similar stained patterns as far as the bole in the region of the active crown was concerned.

#### THE STAINED PATTERN

The dye injected at any point in the bole did not ascend to the top in a straight line parallel to the axis of the tree, but rather traveled in a helical path about the axis. However, these helical pathways were somewhat different in each growth ring. In the inner growth rings, the angle formed between the trace of the dye and the axis parallel was usually most inclined to the left if one observes the stained pattern from the injection upward to the top of the tree. In the younger (outer) sapwood layers, the angle of deviation changed continuously, growth ring by growth ring. It later became more or less straight, and finally was inclined to the right.

These changes in angle, together with enlargement of the growth rings, led to a different winding momentum in the various layers of the sapwood. Consequently, the stained pattern turned faster in the inner xylem than in the outer. The regularity of continuous change in the angle of deviation and of the enlargement of the circumferences of the growth rings made the dyed fibers of each growth ring lie adjacent to the dyed portion of the prior growth ring. As a whole, the various small portions of each stained growth ring appeared as a continuous spiral band over the transverse section (Fig. 4 A, C). With the increased distance from the point of injection as well as the decreasing diameter of the same growth ring towards the tip, the spiraling effect was increased and resulted in additional turns. The typical picture several yards above an injection was the multiple spiral turning clockwise when followed from the outer xylem towards the pith.

In other words, the dye injected through a single hole in the lower bole spread out gradually over the whole transverse section while ascending to the top of the tree. It should be noted that all trees investigated showed the same pattern of spiral distribution at least in the region of the active crown. This extensive distribution above the point where the transverse section of a tree showed a complete spiral turn, provided all limbs with dye. The presence of dye in an older or younger growth ring depended upon the relation of a particular limb to the injection. After a second spiral turn was completed, each limb showed the dye in two different growth rings and so on. Necessarily, limbs towards the top of a tree possessed multiple connections with the stained conduits, whereas a few lower branches were not reached by the dye at all.

#### SLOPE OF GRAIN AND TRANSPORT OF DYE

In general, the transport of the dye followed directly the tracheidal conduits of the xylem. This means that the slope of grain of the wood and the angle in which the stained pattern deviates from a parallel run to the

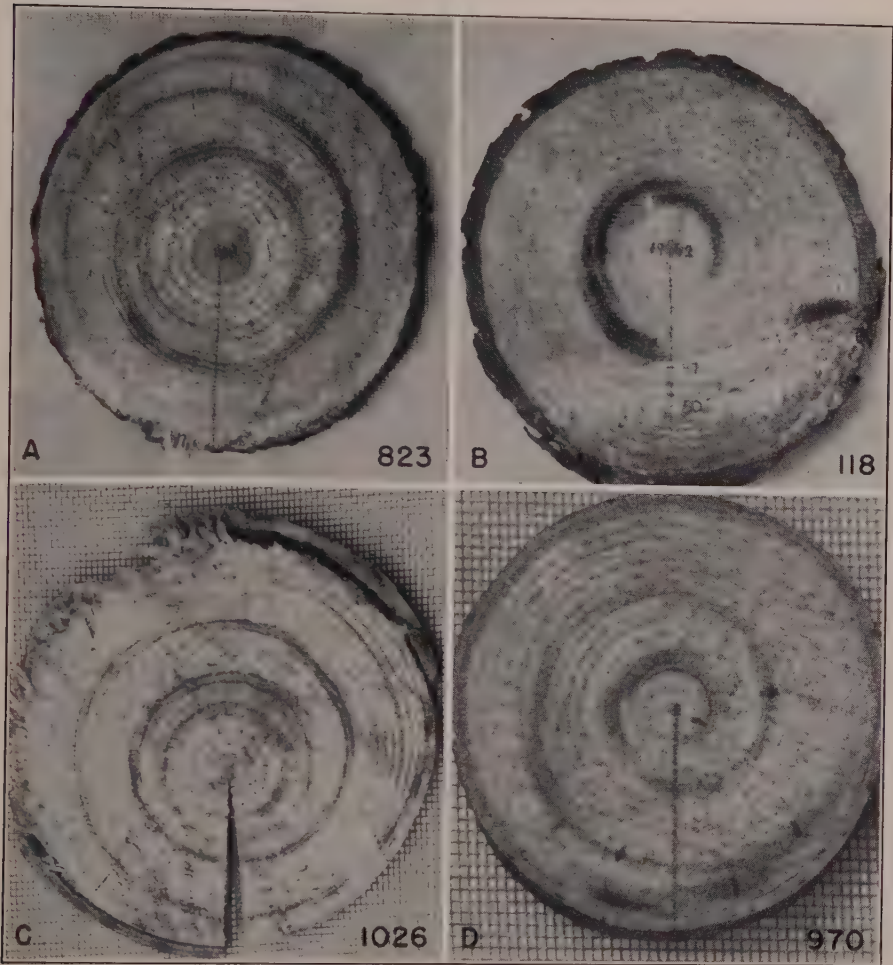


FIGURE 4. Transverse sections through the upper boles of ponderosa pines (A, B) and white firs (C, D) after injection with Acid Fuchsin. The numbers show the distance in centimeters of the transverse section above injection level. (A) Seventy-year-old ponderosa pine; (B) 29-year-old ponderosa pine, bole cut through one-third of circumference prior to injection; (C) 97-year-old white fir; (D) 70-year-old white fir showing reaction to release of dense overstory in change of stained pattern.

stem axis were found to be identical. This is shown by a comparison between the run of the stained pattern and the slope of grain in the same bole by means of the common methods developed for determination of the slope of grain (30, 44). In addition, another simple experiment was helpful in demonstrating this coincidence. When, prior to injection, the bole was cut half or a third through at a level above injection, the stained



pattern was present only in the part of the sapwood that was not disrupted (Fig. 2 B). The unstained section below and above the incision was identical to the portion cut through. In contrast to other methods (12, 13, 14), at least in the conifers investigated, it was found that the *slope of grain was apparently the determining factor in the development of the stained pattern obtained*. This fact makes the procedure developed available for studies on the wood structure of living trees.

#### VARIATIONS ACCORDING TO AGE

The slope of grain differs not only in the various growth rings but also in its vertical run in the same growth ring (4, 5, 9, 27, 32). This fact was shown in the stained pattern, too. Furthermore, the patterns obtained made the existence of certain principles in the arrangement of the tracheidal conduits obvious. Mainly, they showed that the differences in angle of deviation are first of all due to the location in the tree and hence the age of the observed portion of a growth ring (Fig. 3).

As explained earlier, the angle of deviation changes continuously in subsequent growth layers. The inner layers of the sapwood always show a stronger angle to the left than the following layers. This means that a single growth ring in an older tree when observed from the base toward the crown, consists of conduits that may deviate to the right of the stem-axis parallel near the base, then follow the parallel for a while, and then turn to the left in a progressively stronger deviation as they reach the top of the trunk.

No special investigations have been undertaken yet to determine whether or not a one-year-old seedling or the leader of an older tree possesses spiral grain. According to Mayer-Wegelin (27), no seedlings of any tree species show slope of grain, while Northcott (31) recently reported spiral grain in seedlings and the leaders of several tree species. In accordance with these authors and Misra (28), spiral grain was observed in two-year and older seedlings as well as in the second and following growth rings of the tips of older ponderosa pines.

In addition, the trunk of a tree as well as the main stem of the lower crown of an older tree showed repeated changes in direction in different xylem layers. This resulted in a typical stained pattern. In the spiral pattern in the central sapwood each general change in the orientation of the xylem structure was marked by a turn against the direction of the original continuous deviation. Normally, this occurred as a result of changed environmental conditions involving the shape of crown and the width of the annual diameter growth, as will be shown later.

However, these changes did not alter the clear picture of the clockwise spiral dyed in a transverse section through the bole of the upper crown (Fig. 4 A, C). Generally, one can say that the portions of a given age of all

trees investigated showed the same general pattern or tendency. That is, the young tree, the crown of the middle-aged tree, and the top of an older tree turned out to show almost identical dyed patterns in both of the species investigated. Certain specific differences, however, exist between these species (45).

Two examples may explain these findings. A 65-year-old ponderosa pine that had grown poorly showed a slightly decreasing deviation to the left until its 23rd year of growth (1915); later, the xylem grew progressively to the right in the lower part of the trunk. In 1934 the deviation to the right reached a maximum and a decrease in the right orientation followed. However, this situation was found only in the lower bole, whereas the upper bole showed a continuously decreased deviation to the left. A very similar pattern was found in white fir. Figure 3 represents the run of the tracheidal conduits in the growth rings, 1926, 1938 and 1957, of a 97-year-old dominant tree of this species. Each growth ring showed, from the base toward the top of the tree, an increased deviation to the left. Horizontally the inner (older) growth ring showed stronger deviation to the left than the following younger one.

At the same level only slight differences in the angle of deviation were observed within a growth ring. Two or three separate injections made at the same level produced a similar but not quite parallel pattern in each transverse section above or below the dye injections.

#### VARIATIONS ACCORDING TO ENVIRONMENT

An obvious relationship exists between the rate at which the angle of the tracheidal conduits continuously changes and the growth rate of the tree, i.e., poorly growing trees with small growth rings, showed most rapid changes in deviation from the left to the right of the tree axis. The deviation angle in fast growing trees with wide growth rings changes comparatively more slowly in subsequent growth rings.

To substantiate these findings, dye was injected into the lower bole of 18 ponderosa pines selected from a stand of trees of about the same age (compartment 26a, Boyce Thompson Institute Experimental Forest, Grass Valley). According to their diameter at breast height (dbh), the selected trees represented four diameter classes with corresponding height and needle weight (Table II). The trees were sectioned and the dye traced from disks cut from the trunks at 150 and 300 cm. above injection (Fig. 5). The evaluation was confined to the run of the tracheidal conduits in the outer growth ring (1958), in a growth ring of the middle portion of the sapwood (1948) and in a growth ring of the inner sapwood (1943). The average deviation was formed for each selected growth ring and each diameter class separately, and transferred into the coordinate system (Fig. 6).

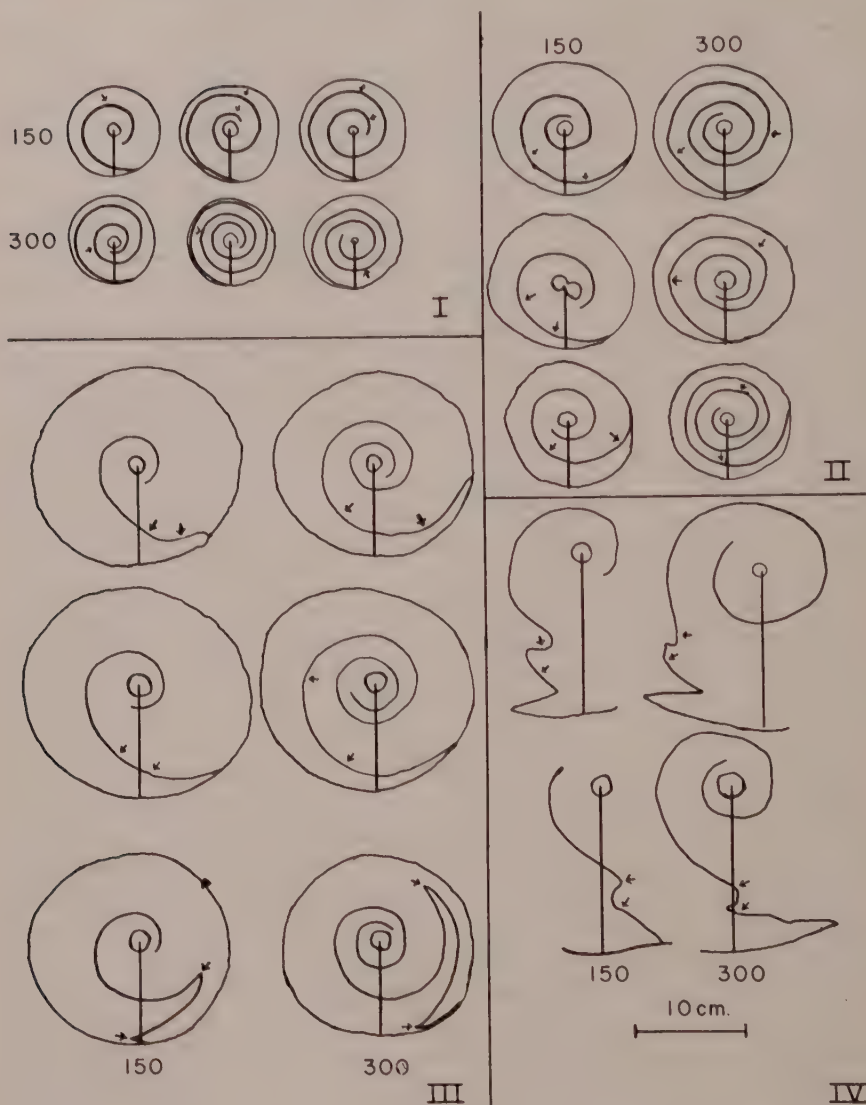


FIGURE 5. Pattern of dye distribution at 150 and 300 cm. above the point of radial injection into the trunks of *P. ponderosa* of comparable ages: I. very small trees, 3 samples (27 years old); II. small trees, 3 samples (32 years old); III. medium trees, 3 samples (32 years old); IV. large trees, 2 samples (33 years old). Given are the outer (1958) and the inner growth ring, and the stained pattern obtained by injection of Acid Fuchsin into the lower bole 110 cm. above ground. The arrows indicate the points at which the stained pattern crosses the growth ring of 1949 and 1944. Further explanation in text, and Table II.

TABLE II  
CLASSIFICATION OF TREES WITH MEANS AND RANGES OF MEASUREMENTS

| Class of trees     | Samples | Age, years    | Diameter breast height, cm. | Height, cm.         | Crown length, cm.   | Needle-weight, kg. | Angle of deviation 1944-1958 |
|--------------------|---------|---------------|-----------------------------|---------------------|---------------------|--------------------|------------------------------|
| I—Very small trees | 5       | 27<br>(24-32) | 11.2<br>(10.5-11.5)         | 1540<br>(1300-1670) | 650<br>(600-870)    | 2<br>(1.1-2.6)     | 6.7                          |
| II—Small trees     | 5       | 32<br>(30-36) | 15.4<br>(14.0-16.5)         | 1720<br>(1500-1850) | 840<br>(700-1000)   | 5<br>(4-6)         | 3.65                         |
| III—Medium trees   | 5       | 32<br>(30-34) | 23.1<br>(21.0-24.5)         | 1960<br>(1700-2300) | 1150<br>(1000-1300) | 20<br>(18-25)      | 2.15                         |
| IV—Large trees     | 3       | 33<br>(33-34) | 37.3<br>(36.5-39.5)         | 2517<br>(2300-2650) | 1200<br>(1100-1350) | 100<br>(84-115)    | 0.25                         |

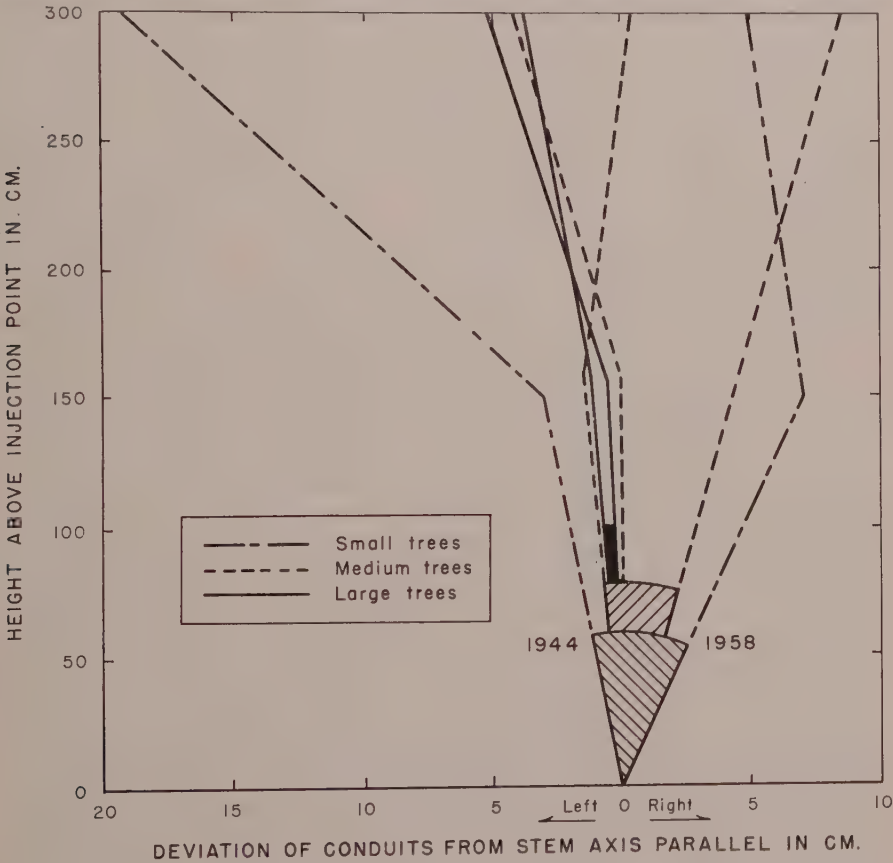


FIGURE 6. The average route of the tracheidal conduits in 32/33-year-old ponderosa pines of different vigor in the growth rings of 1944, 1949 and 1958. O = Point of injection. The angle by which the conduits depart in 1958 from perpendicular in comparison with 1944 is indicated by broken line for small trees, dotted line for the class of medium trees and solid line for the large trees.



Figure 5 represents the dyed pattern observed in 11 trees at the two levels. The multiple spirals in the two classes of small diameter indicate the rapid changes in deviation angle from the left toward the right of the stem axis during the 15 years (1944-1958). The medium-sized trees showed considerable changes, while only slight differences occurred in the larger trees. The angle of declination in wood laid down in the period 1944 to 1956 was  $6.7^\circ$  in the very small trees,  $3.65^\circ$  in small trees,  $2.15^\circ$  in medium trees, and only  $0.25^\circ$  in large trees. These measurements refer to the difference in angle with which the tracheidal conduits deviated from the line perpendicular to the point of injection. These angles of deviation as well as the further run of the tracheidal conduits are given in Figure 6, for small, medium and large trees, which have nearly the same age of 32 (30-36) years. Since the very small trees were considerably younger they were excluded from further consideration.

The same relationship between growth rate and the rate of deviation of the tracheidal conduits may be shown for an individual example. A 70-year-old ponderosa pine with twin boles that had grown at different rates was injected in both trunks at the same level. In spite of genetic equivalence, the trunks showed different arrangement of the tracheidal conduits in the same annular rings (Fig. 7). Again, in all transverse sections observed, the angle in which the tracheidal conduits deviate from the stem axis changes faster in the smaller trunk with a diameter of 25.5 cm. at the injection level than in the larger trunk with a diameter of 31 cm.

Another indication of the close relationship between growth rate and arrangement of the tracheidal conduits was described earlier (44). Heavy natural pruning as well as remaining competition from nearby trees on suppressed trees resulted in changing the pattern of continuous deviation. Sawing off all the branches in the lower crown forced the sudden change of the angle of deviation in the following season or seasons towards the left, whereas releasing reversed the trend of continuous change in angle (Fig. 4 D).

The reason for these changes is not yet understood. In the case of heavy pruning, it seems only logical that the sudden loss of the lower crown, which would occur naturally with aging, leads faster to the same structure or arrangement of the tracheidal conduits in the new growing sapwood layers that would appear gradually in the following years. In the case of releasing, the increased diameter growth in the following years may account for the slowing down and eventual reversal of the rate of continuous change in deviation. Whatever the reasons, one fact is obvious: the angle of deviation or, in terms of wood technology, the slope of grain, is governed by the growth rate, which, in turn, is influenced by the environmental conditions. This may explain the great variation in the xylem structure of individual trees (27, 31) found by wood technologists.

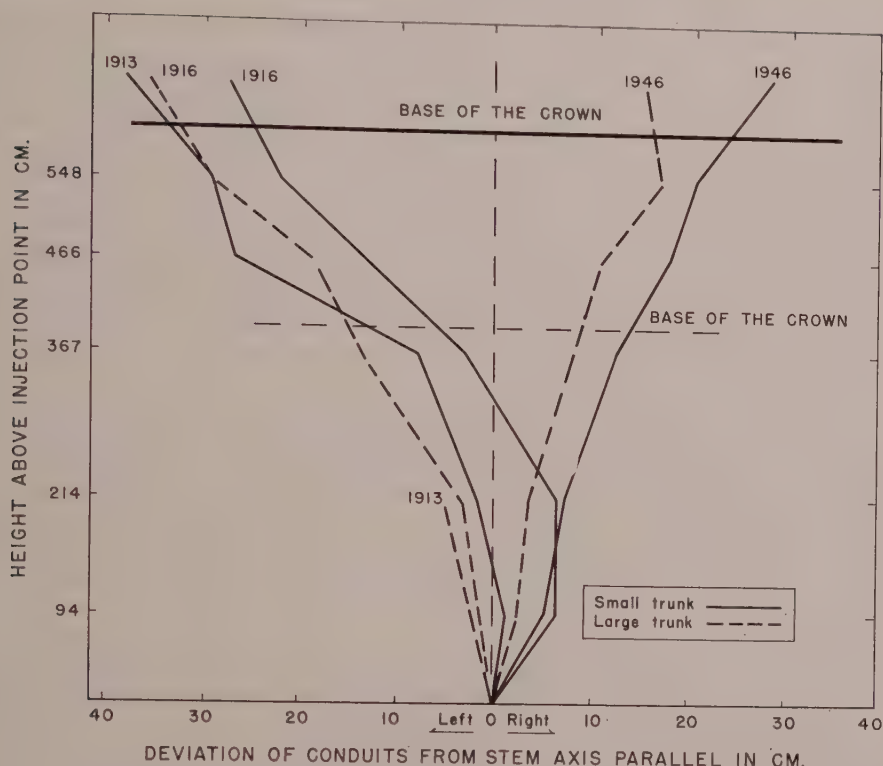


FIGURE 7. Run of the tracheidal conduits in the smaller bole (solid lines) and the larger bole (dotted lines) of a twin tree of ponderosa pine. Otherwise as in Figure 3.

Obviously the same influences are responsible for anatomical changes occurring in the xylem fibers, namely, the angle in which the fibrils in the secondary wall layer of summer wood fibers are inclined against the fiber axis. Paul (32, p. 9) stated that, "another interesting feature found was that when ring width was reduced after heavy pruning for crown control, there was a sudden decrease in fibril angle accompanying the change in ring width" in southern pines. According to Pillow *et al.* (33, p. 7), "the fibril angles in the released trees, which had started as seedlings under hardwoods, tended to decrease in size in the early annual rings similarly to the decrease in the thicket-grown trees. After release from the hardwoods, however, the angles increased abruptly in association with increase in width of the annual rings" in *Pinus taeda* L.

#### CONCLUSIONS

According to the experiments presented here, the dye injected into the sapwood of the lower bole ascends to the crown region along pathways

that are conduits—consisting of vertically adjacent tracheids, because the stained pattern obtained was found to follow the grain or fiber direction of the sapwood. Therefore, the angle in which the stained pattern deviates from a parallel run to the tree axis and the slope of grain are identical. It may be assumed that the transpiration water also follows the same pathways in its axial ascent.

The results of dye injections confirmed the recent concept of several wood technologists (27, 31) that the existence and variation of the slope of grain is the rule rather than the exception in conifers. Furthermore, the dye experiments showed the existence of definite patterns in the arrangement of the tracheidal conduits. For the tree species investigated, these patterns seem to be:

(a) The tracheidal conduits are arranged in a water-conducting system that is basically the same in both species studied. The initial orientation of the tracheids to the left and the continuous change of the angle of deviation toward the opposite side in subsequent growth layers provide decentralized distribution of the material transported from a narrow sector of the tree base over almost the whole crown region. This means that each root is axially connected with all limbs of the upper crown and with most limbs of the lower crown region.

(b) The rate in which the angle of deviation changes from growth ring to growth ring varies individually. These changes are due to environmental conditions involving the growth rate. The continuous change in deviation is retarded as the growth rate increases and accelerated as the growth rate decreases.

These findings lead to the conclusion that in the tree species investigated not only the transport but also the dynamic distribution of the transpiration water over the whole transpiration surface is accomplished by the sapwood.

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# THE WATER-CONDUCTING SYSTEMS IN CONIFERS AND THEIR IMPORTANCE TO THE DISTRIBUTION OF TRUNK INJECTED CHEMICALS

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## SUMMARY

The routes by which water is conducted upward in conifers have been studied by injecting Acid Fuchsin near the base of young to middle-aged trees belonging to 23 species from 12 genera and 4 families. A few days after the dye had been injected, the trees were felled and sectioned to see whether the dye solution had moved directly upward or deviated from the axis of the tree in the several growth rings where it had been incorporated. Five different patterns of translocation were detected in the sapwood of the conifers examined. These five types of water-conducting systems are described, and the way they effect distribution of the injected materials is presented.

Type A. The *spiral ascent, turning right*, is characteristic of the investigated species of *Abies*, *Larix*, *Picea* and subgenus *Diploxylon* Koehne of the genus *Pinus*. As seen in the transverse sections, the fuchsin-dyed strip, which at the place of injection cuts radially across the sapwood, becomes in the higher transverse cuts a spiral band. The spiral runs from the outermost growth ring clockwise toward the center of the stem.

Type B. The *spiral ascent, turning left*, possessed by *Pinus monticola* and *P. lambertiana*, shows the same system as type A, but in a reversed direction.

Type C. The *interlocked ascent* is found in *Sequoia*, *Libocedrus* and *Juniperus*. The dyed radial strip at the place of injection changes into a zigzag pattern that extends over the transverse surface of the stem as the distance increases from the point of injection.

Type D. The *sectorial, winding ascent* appears in *Tsuga* and *Pseudotsuga*. The dye ascends in the same sector along the entire stem, although the sector winds around the stem axis.

Type E. The *sectorial, straight ascent* in *Thuja* and *Chamaecyparis* translocates the dye almost straight up from the point of injection.

The differences in the water-conducting systems usually exist between families or genera but in one instance also within a genus (*Pinus*). Finally some observations are given of specific variations within these systems shown by some characteristics of the conifers studied.

## INTRODUCTION

The ascent of sap in woody plants is associated with longitudinal elements comprising water-conducting layers ("growth rings") in the physiologically active sapwood zone of the xylem. In older trees, the sapwood forms the outer zone of wood encircling the nonfunctional heartwood and may be differentiated from the latter by its lighter color in some species.

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In conifers water transport is effected by hollow tracheids several millimeters long, which form the water-conducting "channels," tracheidal conduits. Besides the axial transport of water in the tracheids, some limited tangential and radial translocation also takes place, but it will not be discussed in this paper. The subject of this study has been exclusively the axial movement of the transpiration stream and the course it takes through the sapwood of various conifers while ascending from the roots of the tree crown.

According to the prevailing but little investigated concept, the transpiration stream ascends from the roots to the branches of the crown by a more or less direct vertical path. Considered in its entirety, the transpiration stream is thought to flow in the entire water-conducting transverse surface of the xylem. Actually, however, this concept is incorrect. The conduit through which any water particle is conveyed is fixed in a system, and the system in which such tracheidal conduits are arranged in a tree stem varies greatly among the conifers. This fact can be proven by the introduction of dyes into the transpiration stream.

The present paper describes for the first time the various structural types of water-conducting systems found in conifers. The spatial course or locality of the transpiration stream in the trunk of some Pinaceae [*Pinus ponderosa* Laws., *Larix decidua* Mill. and *Abies concolor* (Gord. & Glend.) Lindl.] was recently presented (2, 4). Also the age and site influences upon the formation of an individual water-conducting system and the role of the slope of grain in water distribution to the tree crown of *Abies concolor* and *Pinus ponderosa* have been described (3, 4).

#### EXPERIMENTAL METHODS

The investigations presented here began with two particular questions: 1) What is the basic structure of the water-conducting system in various conifers? 2) How is the distribution of stem-injected materials over the tree influenced by the structure of the conducting system? Practical application of the answers to these questions would concern the possibilities of controlling destructive forest insects either by injected chemicals or by greater host resistance against insect attack.

The method used in this investigation has been described recently (4). A 0.3 to 1 per cent solution of Acid Fuchsin, in water or alcohol, was applied near the base of the tree through a radial cut in the sapwood, made either by an injection knife or by a hole bored into the trunk. The injection knife or hole was supplied with dye through a plastic tube from a bottle hung above the place of injection. After one or several days, depending on the size of the tree, the injected tree was sectioned, and sample disks were taken at regular intervals above the point of injection. It was important for the analysis of the stained pattern to mark on each disk the point

directly above the actual place of injection. This prevented a distortion of the original relation of the sample disks to the trunk.

It was learned in previous experiments that a clear picture of the water-conducting system can be obtained best from the crown region (4); therefore, younger trees were usually chosen. As a rule, in each species studied in the present tests young trees (10 to 20 years) as well as middle-aged trees (40 to 80 years old) were taken. The tree species were as follows:

Pinaceae (Pine family)<sup>1</sup>: *Pinus ponderosa* Laws. (ponderosa pine), *P. contorta* Dougl. (lodgepole pine), *P. lambertiana* Dougl. (sugar pine), *P. monticola* Dougl. (western white pine); 56 sample trees. *Abies concolor* (Gord. & Glend.) Lindl. (white fir), *A. lasiocarpa* (Hook.) Nutt. (sub-alpine fir), *A. amabilis* (Dougl.) Forbes (Pacific silver fir), *A. grandis* (Dougl.) Lindl. (grand fir), *A. procera* Rehd. (noble fir); 17 sample trees. *Larix decidua* Mill. (European larch), *L. occidentalis* Nutt. (western larch); 21 sample trees. *Picea sitchensis* (Bong.) Carr. (Sitka spruce), *P. engelmannii* Parry (Engelmann spruce); 8 sample trees. *Tsuga heterophylla* (Raf.) Sarg. (western hemlock), *T. mertensiana* (Bong.) Carr. (mountain hemlock); 5 sample trees. *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir); 4 sample trees.

Taxodiaceae (Redwood family): *Sequoia gigantea* (Lindl.) Decne. (giant sequoia), *S. sempervirens* (D. Don.) Endl. (redwood); 4 sample trees.

Cupressaceae (Cypress family): *Libocedrus decurrens* Torr. (incense-cedar); 16 sample trees. *Thuja plicata* Donn (western redcedar); 4 sample trees. *Chamaecyparis lawsoniana* (A. Murr.) Parl. (Port-Orford-cedar); 4 sample trees. *Juniperus occidentalis* Hook. (western juniper); 2 sample trees.

Taxaceae (Yew family): *Taxus brevifolia* Nutt. (Pacific yew); 2 sample trees.

#### THE WATER-CONDUCTING SYSTEMS

The species investigated can be classified into five types according to the structure of their water-conducting systems.

Three of these types possess well-developed water-conducting systems providing extreme decentralization of the tracheidal conduits. They can be clearly identified and distinguished. Although specific variations in the general pattern are exhibited by certain tree species, the basic structural features of one or the other of the major conducting systems can be identified clearly.

The fourth and the fifth types are made up of all those species that, despite considerable specific variation, show similarities that distinguish them from the other three types. They show less decentralization in water

<sup>1</sup>Scientific and common names are taken from Little (1).



conduction and seem to be far less specialized. More detailed studies are needed to provide a basis for finer classification of these types.

The description of the various types can be given best from the dye-stained pattern revealed on transverse sections. The five types shown in Figure 1 depict schematic portions of typical trunks.

Type A. (Fig. 1 A) The fuchsin-dyed strip, which at the place of injection cuts radially across the sapwood, becomes in the higher transverse sections a spiral band. How the spiral bands occur was described earlier (4). The spiral runs from the outermost annual rings clockwise toward the center of the stem. This characteristic was found in all the investigated species of the genera *Abies*, *Picea* and *Larix* as well as *Pinus ponderosa* and *P. contorta*. This system is designated as the *spiral ascent, turning right*.

Type B. (Fig. 1 B) The same system, but in a reversed direction, is possessed by the white pines, *Pinus monticola* and *P. lambertiana*. On the transverse surface of the stem, the dyed spiral turns counterclockwise from the periphery toward the center of the stem. This system is designated as the *spiral ascent, turning left*.

Type C. (Fig. 1 C) The dyed radial strip at the place of injection changes into a zigzag pattern that extends over the transverse section of the stem as the distance increases from the point of injection. This system was found in both species of *Sequoia* as well as in *Libocedrus decurrens* and in *Juniper occidentalis*. It is designated as *interlocked ascent*.

In Types D and E, the radial extension of the injected material in the crown is not basically greater than at the point of injection. Instead, a disk taken from the crown shows stained patterns similar to that found near the place of injection. Within individual growth rings, however, the dyed sector may extend somewhat tangentially, particularly as the distance increases above injection. In other words, the transpiration stream ascends within the inside layers as well as the outer layers of the sapwood in superimposed parts of the growth rings, thus ascending within only one sector of the crosscut surface. This way of ascent corresponds more or less to the concept of water transport held hitherto.

Among the tree species showing the concentric ascent of the water-conducting channels, one can recognize two different systems.

Type D. (Fig. 1 D) The tracheidal conduits of the inner and outer growth rings remain in the same sector along the entire stem, although the sector winds around the stem axis: *sectorial, winding ascent*, found in *Tsuga* and *Pseudotsuga*.

Type E. (Fig. 1 E) The tracheidal conduits run more or less parallel to the stem axis in all layers of the sapwood: *sectorial, straight ascent*, found in *Chamaecyparis* and *Thuja*.

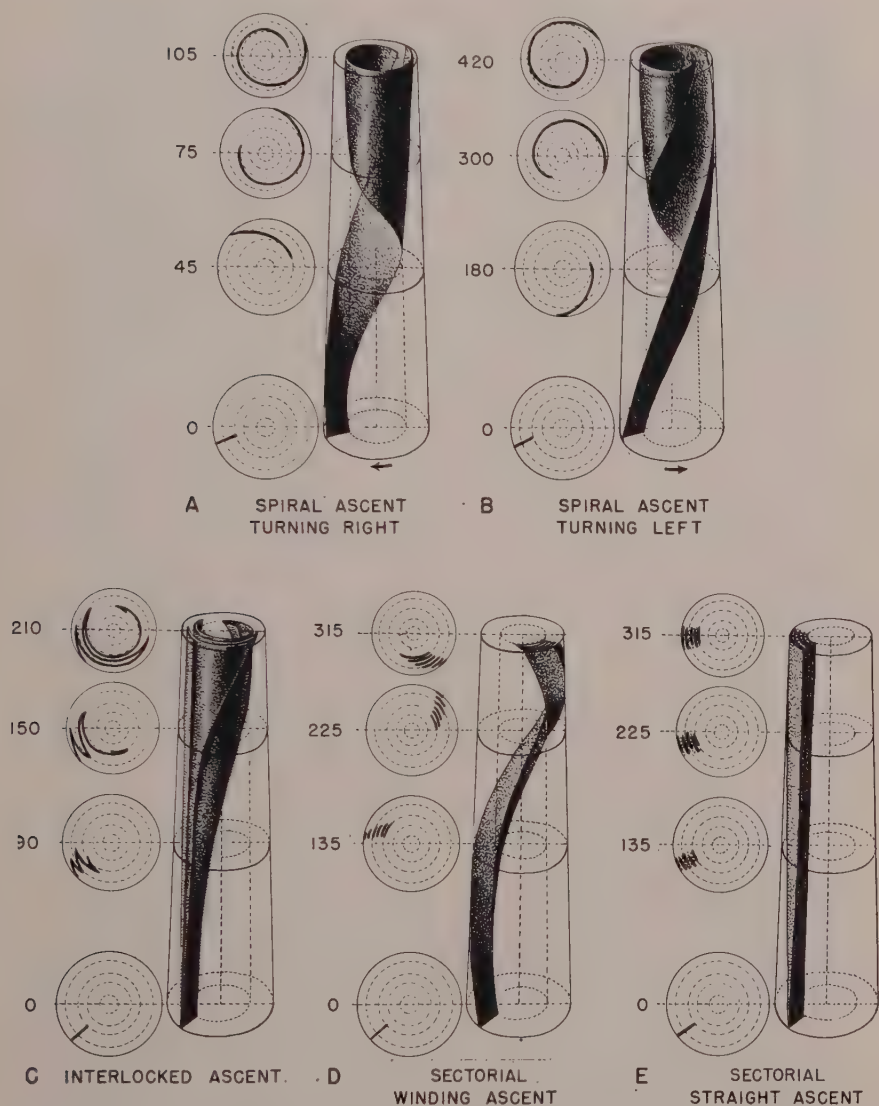


FIGURE 1. Five types of the water-conducting systems in various conifers as shown by the tracheidal conduits that were dyed by trunk injection. The numbers give the height in centimeters of the transverse section above injection. (Slightly schematized.) (A) Spiral ascent, turning right: *Abies*, *Picea*, *Larix* and *Pinus* (*Diploxylon*); specimen, *Larix decidua*. (B) Spiral ascent, turning left: *Pinus* (*Haploxylon*); specimen: *Pinus lambertiana*. (C) Interlocked ascent: *Sequoia*, *Libocedrus* and *Juniperus*; specimen, *Libocedrus decurrens*. (D) Sectorial, winding ascent: *Tsuga* and *Pseudotsuga*; specimen, *Tsuga mertensiana*. (E) Sectorial, straight ascent: *Thuja* and *Chamaecyparis*; specimen, *Thuja plicata*.

## SPECIFIC CHARACTERISTICS

Among the conifers that possess the same conducting system, individual genera or species may exhibit additional characteristics. Thus, certain tree species are characterized by a very regular, often mathematically exact arrangement of their tracheidal conduits. This is particularly true in all the species of the genus *Abies* studied, which have a very regular water-conducting system. The regular and gradual shift of the tracheidal conduits from growth ring to growth ring in *Abies concolor* results in a very uniform spiral band (Fig. 2 B). Even transverse sections from below the active crown frequently exhibit the regular arrangement. An additional characteristic of this genus is excellent intake of the dye and the ability to transport it far.

Other species tend to sudden change in the deviation angle. Characteristic of *Pinus ponderosa* (Fig. 2 A) are irregular but continuous changes in the slope of the tracheidal conduits in the successive layers of the sapwood. A similar change is shown by *Larix decidua*, whereas in *Larix occidentalis* (Fig. 2 D), *Pinus contorta* and the genus *Picea* (Fig. 2 C) no specific characteristics could be found.

The white pines examined, *Pinus lambertiana* and *Pinus monticola*, are similar to the genus *Abies* in their uniform arrangement of tracheidal conduits, with the difference that they run in the opposite direction (Fig. 2 E, F). Another characteristic may exist in the fact that the spiral band ascends very gradually, i.e., the distance necessary to complete one revolution is considerably longer than in species with right-turning spiral ascent.

A completely different conducting system is present in the species grouped in type C, the interlocked ascent. *Libocedrus decurrens* was studied in some detail as a representative of this type. In this species the radial strip at the injection extends in the upper transverse sections into a zigzag band, as a result of the alternately turning fiber run, "interlocked grain." In other words, the tracheidal conduits ascend to the right in one growth ring, and in the next ring they ascend to the left; correspondingly, the dye is transported to the right side in one ring and to the left in another. Apparently, however, there is no regularity in the direction or the degree of deviation from the tree axis. Thus, they may run for one or more years toward the one side or the other. When the tracheids diverge in the same direction from the parallel of the stem axis for several successive growth rings, this deviation usually occurs with different angles. Only seldom are successive tracheidal conduits arranged parallel to each other. More often even the angle of deviation within the same growth ring is changed. To determine whether the changes in the tracheidal arrangement depend on other than individual influences, five neighboring incense-cedars were injected. They showed completely different directions and deviation angles

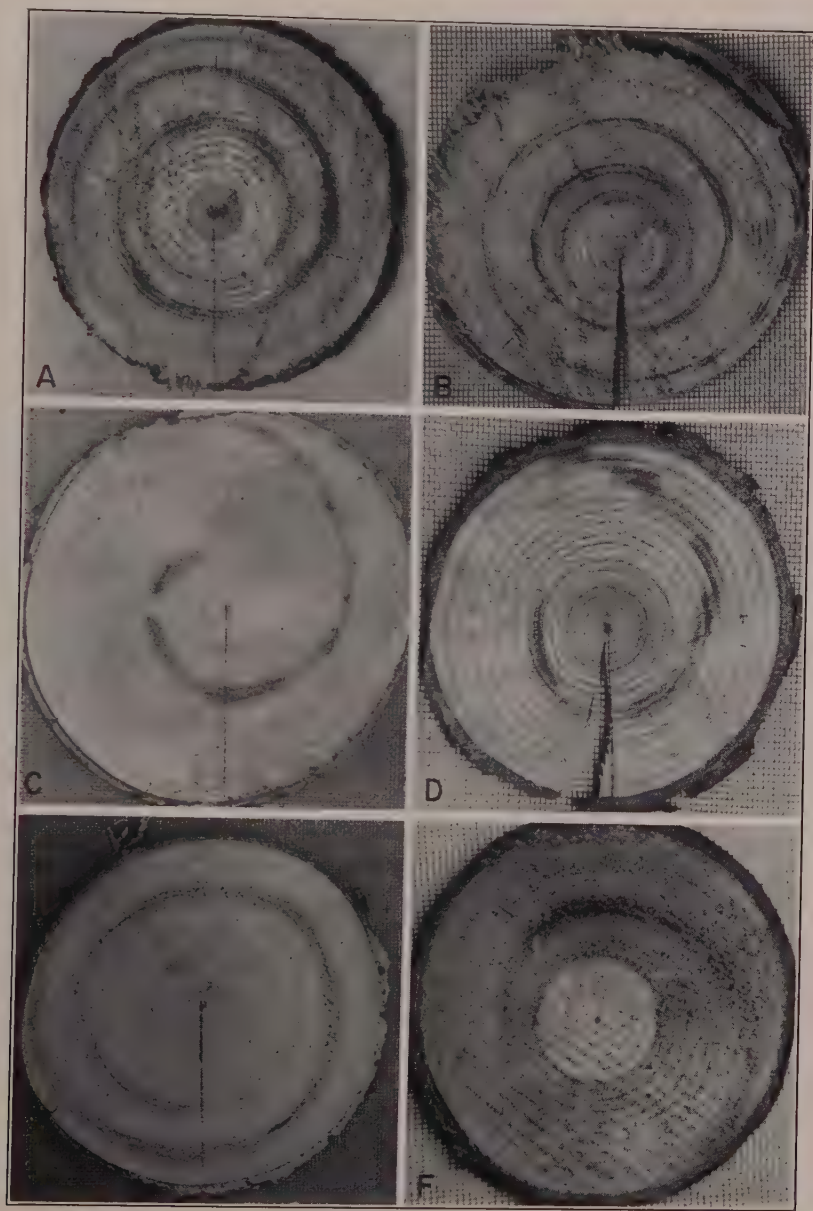


FIGURE 2. Stained pattern resulting from trunk injection of Acid Fuchsin into various Pinaceae. Spiral ascent, turning right: (A) *Pinus ponderosa* (transverse section at 823 cm. above injection level). (B) *Abies concolor* (1026 cm.). (C) *Picea engelmannii* (370 cm.). (D) *Larix occidentalis* (370 cm.). Spiral ascent, turning left: (E) *Pinus monticola* (545 cm.). (F) *Pinus lambertiana* (380 cm.). The radial strips on the transverse sections give the projected position of the injection. As background a  $\frac{1}{16}$ " mesh screen was used.



in the xylem layers of the corresponding growth rings. The expected individuality of the tracheidal organization in a single stem was thus shown, though it remains within the clearly discernible system. The direction and the angle of deviation in corresponding xylem layers of the same tree are similar, however, when one considers the various sides of the same transverse section. This can be proved simply by applying several injections around the stem on the same level. These double or triple injections were done with Acid Fuchsin or with eosin and Acridine Red.

With increased height above the point of injection, the zigzag band spreads over a larger part of the transverse surface of the stem, and later on in the upper crown region it covers the entire surface. The stained

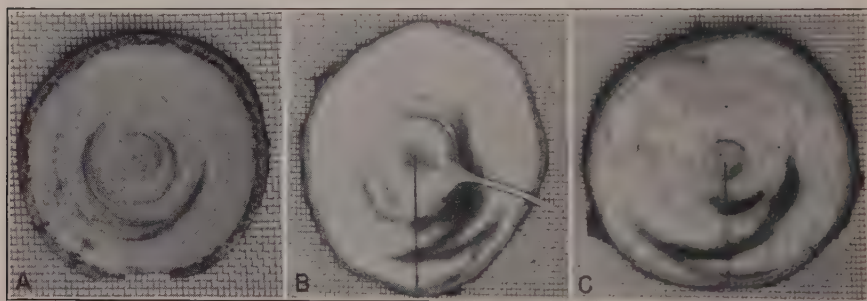


FIGURE 3. Interlocked ascent: (A) *Libocedrus decurrens* (148 cm. above injection). (B) *Juniperus occidentalis* (90 cm.). (C) *Sequoia gigantea* (110 cm.). Otherwise as in Figure 2.

pattern usually extends over one-third or one-half of the transverse section at the base of the crown. Almost never during these experiments were the stained patterns in the various xylem layers of the same intensity. Usually the color was concentrated at the extremities of the stained band and was weaker in the middle portions. This phenomenon can scarcely be attributed to the application of dye by boring a hole, since it was observed also when the dye was applied by an injection knife. It may be due to the immediate change in the direction and angle of the tracheidal conduits in the successive xylem layers.

The same type of water-conducting system was found in *Libocedrus decurrens* (Fig. 3 A), in *Juniperus occidentalis* (Fig. 3 B), and in both species of *Sequoia* (Fig. 3 C); specific characteristic variations were not observed in the several species studied in this group.

Certain tree species with the sectorial ascent of the stained pattern exhibit distinct differences. Typical representatives of the two water-conducting systems are presented in Figure 4. The common characteristic of both systems is the transport of injected material through the adjacent

parts of the various sapwood layers. Here, the dye is not distributed over the entire crosscut surface, but instead it ascends through only one sector. With increased distance from the injection, certain shifts within one or several growth rings may take place, but ultimately they do not change the character of the sectorial ascent.

A more important difference, however, justifies dividing these species into two types of water conduction. This difference lies in the fact that the tracheidal conduits in certain species run from the base of the stem

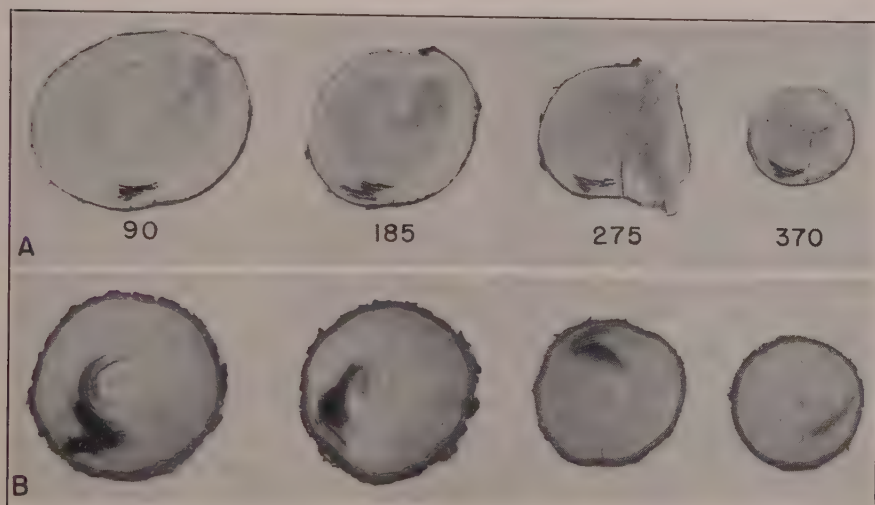


FIGURE 4. Upper row: sectorial, straight ascent. Transverse sections through a western redcedar (*Thuja plicata*) at 90, 185, 275 and 370 cm. above injection level. Lower row: sectorial, winding ascent. Transverse sections through a mountain hemlock (*Tsuga mertensiana*) at the same distances. (The stained pattern in the upper row was slightly darkened by pen.)

to the crown more or less parallel with the stem axis, but in the others they wind around the stem axis. The straight ascent of the dye was found in the Cupressaceae (*Thuja plicata* and *Chamaecyparis lawsoniana*). In *Tsuga* and *Pseudotsuga* the ascent takes place in a winding pattern. Thus the young Douglas-firs always show the tendency to add the new conduits in a winding pattern to the left. Here, however, the tracheidal conduits of individual growth rings may show independence. The dyed parts of individual rings were still within the same sector of the transverse section, yet they sometimes shifted in such a way that the dyed portion of one ring was either slightly ahead or behind the neighboring ring in the direction of the winding ascent. The outer layers of the sapwood in older Douglas-firs, on the other hand, often showed a winding to the right in

successive tracheids. The relationship here could be similar to that found in the age-caused change in deviation of the tracheidal conduits in other conifers (4). The tangential expansion of the dyed tracheidal conduits in individual growth rings is conspicuous, as can be seen also on *Tsuga mertensiana* in Figure 4.

A similar situation was found in *Tsuga heterophylla*. In addition, in this species, another characteristic occurred. At some distance above the injection, the concentric, stained pattern extended in some of the specimens investigated into a diagonal band; somewhat higher, however, the stained pattern concentrated again on a narrower sector of the transverse section. Only more extensive studies will explain this phenomenon, as well as the somewhat similar situation found in *Taxus brevifolia*.

#### THE DISTRIBUTION OF TRUNK-INJECTED MATERIALS TO THE CROWN REGION

The distribution of an injected material to the crown region depends, in the first place, on the conducting system each conifer possesses. The most complete distribution is in the tree species with spiral ascent of sap. Here each twig that is located above the first completed spiral is supplied directly with the injected material. This occurs first of all in one of the growth rings, either younger or older according to the place where the twig strikes the stained pattern. Higher in the trunk, after the completion of a second spiral, even two rings of the twig, one younger and one older, conduct dye. In the upper crown, the twigs are connected manifoldly with the injection. As can be seen in a 60-year-old *Pinus ponderosa* in Figure 2 A, at a distance of 823 cm. above the injection and 1600 cm. below the tip of the crown, each twig is already being supplied from the same injection by three growth rings, and partially even by four. More intensive yet is the supply of the crown terminal. Because in middle-aged and older trees the first spiral is usually completed below the active crown, theoretically the injected material can be distributed to every branch and twig. Quantitatively, this distribution is naturally quite different, depending upon whether a twig is connected with the dye-conducting tracheids of a younger or an older growth ring. In younger trees, on the other hand, a limited region of the lower crown is not supplied at all (Fig. 5).

In the white pines investigated the situation is similar. The slower winding momentum of this type of water conduction (spiral ascent, turning left), however, resulted in the distribution of dye into most of the branches only by the middle region of the crown.

The pattern of distribution is quite different in the species with interlocked ascent. Here the distribution of injected material is very fast and intensive in several growth rings of the lower crown, but only in the sector that was hit by the injected substance. Accordingly, only branches con-

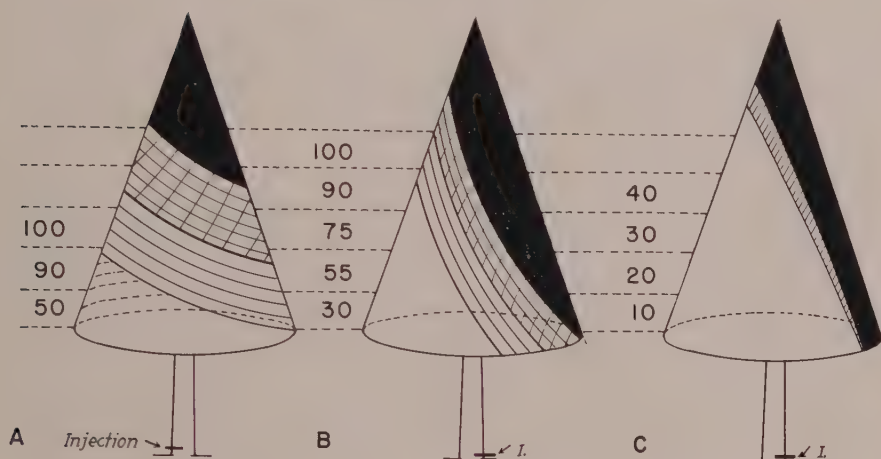


FIGURE 5. Schematized presentation of the distribution of injected dye in the crown region of various conifers. Distribution found in trees with spiral ascent, turning right (A), interlocked ascent (B), and sectorial, straight ascent (C). The figures give the percentage proportions of the branches supplied by the dye in various parts of the crown. The branches connected with the dye injection by one growth ring are represented by a simple lined field, those connected by two rings are shown by a crosslined field, and branches connected by several rings are shown as a dark field.

nected with this sector are supplied. As the dye is spread through the tracheidal conduits over the transverse surface, the number of branches supplied by the injection also increases. After the zigzag bands in the upper crown region have spread over the entire transverse surface and have closed, all the branches are finally supplied. This always occurs below the tip of the crown and shows again the preferred and intensive distribution to the crown tip (Fig. 5).

In the species with sectorial ascent of the tracheidal conduits, only a few branches are supplied with the injected material, i.e., those branches that originate from the narrow dyed sector of the transverse cut. Quantitatively, the transportation is very intensive. The tangential expansion of the stained patterns and their insignificance shifting to one or the other side assure the distribution to the entire crown tip. Also in these species, any injection will reach the crown tip irrespective of the trunk side on which it is applied.

All the conducting systems supply the crown tip, but in different ways. It should be emphasized that the present experiments were concerned only with the study of the pathways by which the water stream travels upward. The quantity of water transported along those ways is another matter. This question is very closely connected with that of the diffusion pressure deficit and, consequently, the quantity of water transported depends on



daytime fluctuations. Moreover, the study presented here suggests that both also vary locally within a single tree. Further information is required to elucidate the significance of the local ascent of the transpiration stream for practical utilization in trunk injection.

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# THE APPLE REPLANT PROBLEM. I. THE EXCRETION OF PHLORIZIN FROM APPLE ROOT RESIDUES

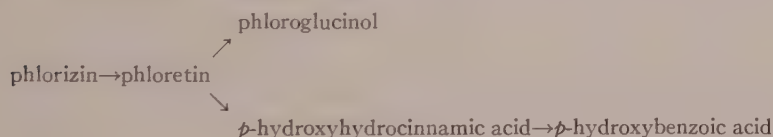
HORST BÖRNER<sup>1</sup>

## SUMMARY

Experiments were carried out to investigate the causes of the apple replant problem from the point of view of a possible action of substances released from residues into the soil. It was found that an addition of dried apple root bark to water cultures produced a strong reduction in growth of apple seedlings. As little as 1 g. of root bark per 500 ml. nutrient solution caused a considerable effect.

Paper chromatograms revealed five phenolic substances from bark held in nutrient solutions for 33 days. The same compounds are present in cold-water extracts from soils containing apple root residues.

Of the five phenolic substances present in nutrient solution and water extracts of soil only phlorizin could be identified as a natural constituent of the bark and wood of apple roots, although quercitrin was also detected. The other four phenolic substances were detected in soils within 2 to 10 days after pure phlorizin was added to different soils. It is quite obvious, therefore, that these substances were decomposition products of phlorizin. The detected compounds were identified as phloretin, *p*-hydroxyhydrocinnamic acid, phloroglucinol and *p*-hydroxybenzoic acid. The breakdown of phlorizin in soils in all probability occurs as follows:



The identified chemicals retarded the growth of apple seedlings in water culture at a concentration as low as  $10^{-4}M$ .

The inhibitory effect on root development was very obvious. The effectiveness of the compounds varied widely. The strongest inhibition was caused by phlorizin and phloretin, while phloroglucinol had only a slight influence.

To what extent the identified chemicals participate in the apple replant problem is not clear at this time. Further investigations are necessary in order to find out the amount of root residues left in the soil and the concentrations of the detected compounds under natural conditions.

## INTRODUCTION

The phenomenon of "soil sickness" in fruit culture is a problem of economic importance in many areas of the world. In Germany, the nursery-

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men encountered this problem after a cultivation of apples for one or two years. They find it necessary to plant apple seedlings in soils which have never before produced apple trees (6, 9, 11, 13, 27). Similar difficulties with peach (15, 24) and citrus (18, 19, 20) have been reported from Canada and California. The replant problem is, therefore, widespread.

The symptoms on affected apple trees, growing in old orchard sites, are usually manifested by retarded growth and shortened internodes resulting in a rosette-like appearance. Furthermore, the roots show varying degrees of discoloration and a reduced growth of the tap root. These symptoms are reversed when the apple seedlings are shifted from toxic to normal soil. Apple seeds germinate as readily in affected soil as in normal fields (6, 9, 13, 27). The primary cause of the apple "soil sickness" has not been fully explained even though intensive investigations have been made. The existing publications state that, besides an impoverishment of soils of microelements (14) and a damage of apple roots by nematodes (22, 23, 30), the root residues (9, 27) remaining in the soil play an important role in this problem. Root residues do have an effect on apple replants as has been shown by Fastabend (9). He revealed that chopped apple roots or water leached through the affected soils produced the toxicity symptoms in seedlings when added to normal soil. But no investigator dealing with this problem has succeeded in identifying the toxic substances.

In the present paper the apple "soil sickness" problem was investigated exclusively from the point of view of a possible action of substances which are given off from root residues, with the main interest devoted to the identification of the toxic principles.

#### INFLUENCE OF ROOT BARK ON THE GROWTH OF APPLE SEEDLINGS IN WATER CULTURE

*Materials and methods.* Root bark of 16-year-old apple trees was added to water cultures of apple seedlings prepared as follows. The outsides of 500 ml. Erlenmeyer flasks were coated with white paint. The flasks were filled with a nutrient solution shown by Kobernuss (14) to be adequate for the growth of apple seedlings. This solution contained water, 1000 ml.;  $\text{KNO}_3$ , 1.0 g.;  $\text{Ca}_3(\text{PO}_4)_2$ , 0.25 g.;  $\text{Fe}_3(\text{PO}_4)_2$ , 0.25 g.;  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.50 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.50 g.; and the minor elements  $\text{H}_3\text{BO}_3$ , 0.02 g.;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.01 g.;  $\text{ZnSO}_4$ , 0.001 g.;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.001 g. In all tests 0.001 g. ferric disodium ethylenediamine tetraacetate containing 9 per cent iron was also added to each liter of the nutrient. Each flask contained one apple seedling supported by a cotton collar in a hole in a paraffined cork. The culture was aerated by compressed air injected through a glass tube inserted into the cork and drawn to capillary size inside the flask.

Apple (*Malus baccata* Borkh., Siberian crabapple) seedlings were obtained from seeds soaked for 12 hours in distilled water, held for 28 days

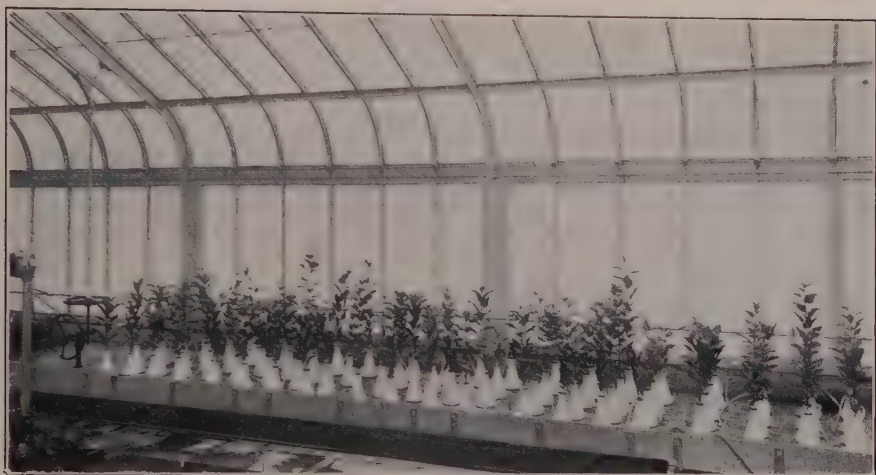


FIGURE 1. Water culture arrangement for testing the effect of different substances on the growth of apple seedlings.

in moist granulated peat moss at 5° C. (7, 10, 26) and sown in moist sand in the greenhouse. As soon as the seedlings developed first leaves they were used for the water cultures.

One water culture experiment consisted of 16 flasks, which were divided into 4 rows of 4 flasks each (Fig. 1). The flasks of the first row were used as controls and contained only nutrient solution. To each flask of the second, third and fourth rows were added 0.2, 1 and 10 g., respectively, of root bark from a 16-year-old apple tree (*Malus sylvestris* Mill., variety Stayman Winesap, growing on its own roots). The experiment was terminated after 33 days and measurements were made of growth response obtained: length of seedlings (cm.), length of roots (cm.), number of leaves, dry weight of stems (mg.), dry weight of leaves (mg.) and dry weight of roots (mg.). Standard errors were calculated using the formula

$$m = \pm \sqrt{\frac{\sum(x - \bar{x})^2}{n(n - 1)}}.$$

*Results.* The addition of root bark produced a strong growth inhibition of the apple seedlings (Table I and Fig. 2). The root growth was especially affected but the other parts of the plants were also obviously retarded even at a concentration of 0.2 g. in 500 ml. of solution. With as much as 10 g. bark in each flask, the seedlings died within 5 to 10 days.

The toxic effect might be due to substances liberated from the root bark or to an increased development of microorganisms in the nutrient solution. Preliminary tests on the nutrient solution containing 10 g. bark showed there were ether-soluble substances that gave a yellow-red color reaction



TABLE I

INFLUENCE OF ROOT BARK OF A 16-YEAR-OLD APPLE TREE ON THE GROWTH OF APPLE SEEDLINGS IN WATER CULTURE. DURATION OF EXPERIMENT 33 DAYS

| Growth response           | Growth with standard error in flask (500 ml.) containing different amounts of bark |              |              |           |
|---------------------------|------------------------------------------------------------------------------------|--------------|--------------|-----------|
|                           | 0 g.                                                                               | 0.2 g.       | 1.0 g.       | 10.0 g.   |
| Number of leaves          | 13.0 ± 0.9                                                                         | 9.8 ± 0.8    | 7.8 ± 0.9    | No growth |
| Length of stem, cm.       | 16.1 ± 3.4                                                                         | 10.7 ± 2.8   | 4.1 ± 0.3    | No growth |
| Length of root, cm.       | 19.4 ± 0.6                                                                         | 9.9 ± 0.7    | 5.7 ± 0.3    | No growth |
| Dry weight of leaves, mg. | 715.0 ± 65.9                                                                       | 337.5 ± 69.5 | 141.3 ± 29.9 | No growth |
| Dry weight of stem, mg.   | 220.0 ± 57.2                                                                       | 110.0 ± 35.8 | 35.0 ± 6.4   | No growth |
| Dry weight of roots, mg.  | 155.0 ± 9.6                                                                        | 96.3 ± 7.1   | 70.0 ± 9.3   | No growth |

with diazotized sulfanilic acid, thus indicating the occurrence of phenolic compounds. On the other hand, a remarkable increase of the microorganisms in the flasks with root bark was also observed. An effort was made to minimize the influence of the microorganisms by daily destruction of the moldy surface of the solution and washing of the roots with distilled water. In spite of this treatment there is still the possibility that the microorganisms may have contributed to the inhibitory effects.

#### LIBERATION OF PHLORIZIN FROM APPLE ROOT RESIDUES AND ITS DECOMPOSITION

##### DETECTION OF PHENOLIC COMPOUNDS IN WATER CULTURE BY MEANS OF PAPER CHROMATOGRAPHY

As noted above it was possible to detect phenolic compounds in water culture containing apple root bark. In order to separate these by means of paper chromatography ether extracts were made of the water culture solution at the close of the experiment (33 days). Solutions containing 10, 1 or 0.2 g. of bark (500 ml. of first and 2000 ml. of the second and third) were acidified with hydrochloric acid, shaken twice with 1000 ml. ether, the ether evaporated in vacuo, and the residue dissolved in 50 ml. ethanol. The alcoholic solutions could be used immediately for paper chromatography.

*Technique of paper chromatography.* For all experiments Whatman No. 1 paper (size 28×44 cm.) was used. Spots of 20  $\mu$ l. and 40  $\mu$ l. were placed on the paper. The compounds were separated by the ascending method by means of the following solvents: A. acetic acid—water, 15:85 (v/v); B. 20 per cent potassium chloride; C. acetone—formic acid—water, 5:15:80 (v/v/v); D. butanol—acetic acid—water, 3:2:95 (v/v/v); E. butanol—ammonia (butanol saturated with 1.5*N* NH<sub>3</sub>). The detection of the different substances was carried out by aid of the following reagents: 1) 10 per cent solution of sodium carbonate; 2) 1 per cent methanolic solution



FIGURE 2. The influence of apple root bark on the growth of apple seedlings in water culture. Concentration from left to right: control; 0.2; 1; and 10 g. root bark per 500 ml. nutrient solution. Photographed after a culture duration of 32 days. A. Seedlings in culture. B. Corresponding excised roots.

of aluminum chloride; 3) 100 mg. diazotized sulfanilic acid in 20 ml. of 10 per cent sodium carbonate solution; 4) two solutions made up as follows: 25 ml. of a 0.3 per cent solution of *p*-nitraniline in 8 per cent hydrochloric acid are mixed before application with 1.5 ml. of a 5 per cent sodium nitrite solution, and a 20 per cent solution of sodium carbonate. The two solutions were applied one after another (5); 5) fluorescence of the different compounds on filter paper after treatment with ammonia or  $AlCl_3$  (wave length  $\lambda = 366$  and  $\lambda = 256$ ).

*Results.* In nutrient solution containing 10 g. root bark of a 16-year-old apple tree four (I-IV) or possibly five (V), phenolic compounds were detectable. The different substances were separated in solutions A and B (see above) and made visible on the paper by color reagent 3. In nutrient solution containing 1 g. bark, however, only two substances (III and IV) could be detected, while in the flask with 0.2 g. root bark a phenolic compound could be demonstrated only by a spot test. In this case the concentration of the compounds was so weak that separation by paper chromatography was not possible (Table II).

#### LIBERATION OF PHENOLIC COMPOUNDS FROM APPLE ROOT RESIDUES

It has already been shown that phenolic compounds are given off from root bark in the nutrient solution in water cultures. Also it has been mentioned that Fastabend (9) demonstrated that it is possible to produce the "soil sickness" with water washed through soils which contained apple root residues. Experiments to determine whether such soil leachates would also contain the detected phenolic compounds are reported below.

*Materials and methods.* In order to get conditions similar to those in natural soils 25 g. of intact, air-dry apple roots with a diameter of 0.2 to

TABLE II  
DETECTION OF PHENOLIC COMPOUNDS IN WATER CULTURES TO WHICH APPLE ROOT BARK IN DIFFERENT CONCENTRATIONS WAS ADDED†

| Compound No.* | Rf values in solution |      | Color reaction | Detection in nutrient solution to which apple root bark was added† |      |         |
|---------------|-----------------------|------|----------------|--------------------------------------------------------------------|------|---------|
|               | A**                   | B**  |                | 10 g.                                                              | 1 g. | 200 mg. |
| I             | 0.56                  | 0.21 | Yellow         | +                                                                  | —    | —       |
| II            | 0.28                  | 0.02 | Yellow         | +                                                                  | —    | —       |
| III           | 0.51                  | 0.78 | Red            | +                                                                  | +    | —       |
| IV            | 0.65                  | 0.47 | Yellow         | +                                                                  | +    | —       |
| V             | 0.70                  | 0.68 | Yellow         | (+)                                                                | —    | —       |

\* Later identified as I=phlorizin, II=phloretin, III=*p*-hydroxyhydrocinnamic acid, IV=phloroglucinol, V=*p*-hydroxybenzoic acid.

\*\* Solution A, acetic acid—water (15:85), solution B, 20 per cent potassium chloride.

\*\*\* Diazotized sulfanilic acid.

† (+)=Trace; +=compound detected; —=compound not detected.

0.6 cm. were added to 500 g. air-dry soil. The soil was mixed with distilled water and placed in a glass funnel with glass wool. At intervals of from 1 to 3 days, water was added so that 500 ml. of solution leached through the soil within 2 hours and were collected in a separate flask. The leached water was evaporated to 10 ml. in vacuum at 50° C. After cooling, ethanol was added making a total volume of 25 ml. The precipitate present was removed by filtration through filter paper. In another method the solution was prepared for paper chromatography by shaking the evaporated acidified soil extract twice with 50 ml. ether. The ether was evaporated in vacuo and the residue redissolved in 10 ml. ethanol. From these solutions 20 to 40  $\mu$ l. were applied to the paper chromatogram.

The separation took place in solutions A and B, and the compounds were identified by color reaction on the paper with reagent 3. The experiments were repeated three times. Root material from both 6- and 16-year-old apple trees was used.

*Results.* After washing out the soil immediately after adding the root material, the evaporated water contained phenolic substance I. Soil leached 1 to 2 days later yielded compounds identical with the substances II to IV entered in Table II. With increasing length of time, however, substances I and II disappeared and only III and IV were still detectable (Table III). From the color intensity and size of the spot it seems that the concentration of III was greater than that of compound IV. There is, apart from a little difference in the time when compounds appear, no difference between the roots used.

These experiments, therefore, also demonstrated that in soil containing apple root residues, the same substances (except compound V) are detectable as were found previously in water cultures containing apple root bark.

TABLE III

DETECTION OF PHENOLIC COMPOUNDS IN WATER, LEACHED THROUGH SOIL, TO WHICH APPLE ROOTS WERE ADDED

| Compound No.* | Washing out of the soil after days† |       |     |     |     |       |       |     |
|---------------|-------------------------------------|-------|-----|-----|-----|-------|-------|-----|
|               | 0                                   | 1     | 2   | 4   | 6   | 8     | 10    | 13  |
|               | a** b**                             | a b   | a b | a b | a b | a b   | a b   | a b |
| I             | +                                   | +     | +   | +   | +   | — (+) | — (+) | — — |
| II            | — —                                 | +     | +   | +   | +   | — —   | — —   | — — |
| III           | — —                                 | — —   | +   | +   | +   | +     | +     | +   |
| IV            | — —                                 | (+) — | +   | +   | +   | +     | +     | +   |

\* Later identified as I=phlorizin, II=phloretin, III=*p*-hydroxyhydrocinnamic acid, IV=phloroglucinol, V=*p*-hydroxybenzoic acid.

\*\* a=Roots of a 16-year-old apple tree, b=roots of a 6-year-old apple tree.

† (+)=Trace; +=compound detected; —=compound not detected.



## DETECTION OF PHLORIZIN AND QUERCITRIN IN ALCOHOLIC ROOT EXTRACTS

In order to determine whether the detected compounds are natural constituents of the apple root or partly microbial transformation or decomposition products, roots, root wood and bark of two different species of apple were extracted and the extracts separated by means of paper chromatography.

All root material was extracted with ethanol in a Soxhlet apparatus for 48 hours, evaporated in vacuum and filtered after storing for 24 hours in a refrigerator. All extracts could be used for paper chromatography without further preparation. Eighty grams of air-dry root wood (E 1) and 20 g. of air-dry root bark (E 2) of a 16-year-old tree, *Malus sylvestris*, variety Stayman Winesap, were extracted with 500 ml. ethanol each and then evaporated to 100 ml. Four grams of air-dry whole roots of a 6-year-old apple tree (E 3), *Malus sylvestris*, variety unknown, and 12 g. of fresh entire roots of a 3-month-old apple seedling (E 4), *Malus baccata*, were extracted with 60 ml. of ethanol and evaporated to 25 ml.

Phlorizin is a well-known constituent of apple roots (8) and can be readily identified by an infrared spectrum<sup>2</sup> and by paper chromatography (Table IV).

The only other material revealed by reagent 3 was the glycoside, quercitrin, which was further identified by decomposition into its sugars and aglycons. This was achieved for both glycosides by adding  $\text{H}_2\text{SO}_4$  to make the extracts 2*N* and holding at 100° C. for one hour. The aglycons, phloretin and quercetin, were extracted from the hydrolyzed product with acetic ether as described by Linskens (16, p. 218) and were identified by means of paper chromatography (Table IV).

Identification was confirmed by eluting phlorizin and quercitrin after paper chromatographic separation in acetone—formic acid—water (5:15:80, v/v/v; descending method) with a water-ethanol solution (2:1) from the paper and preparation of the eluates in the same way as outlined before. Though there are only small differences between the  $R_f$  values of phlorizin and quercitrin, respectively, and phloretin and quercetin, the different substances can be easily distinguished by their color reactions. Upon spraying the chromatogram with  $\text{Na}_2\text{CO}_3$  the positions of quercitrin and quercetin are easily detectable by their color reaction, while other compounds are not revealed with this reagent. Results are similar when the paper is sprayed with  $\text{AlCl}_3$ . It is also possible to distinguish the chemicals under the ultraviolet lamp after spraying with  $\text{AlCl}_3$  (Table IV).

While phlorizin was detectable in all extracts, quercitrin could be identified only in the root bark and root wood of the 16-year-old apple tree. It is not quite clear at this time whether or not the differences are a matter of the apple species or of the age of the trees.

<sup>2</sup> Mr. William R. Smith kindly conducted the infrared analyses.

TABLE IV

Rf VALUES AND REACTIONS FOR THE DETECTION OF PHLORIZIN, QUERCITRIN AND DECOMPOSITION PRODUCTS

| Solutions*                                                  | Phlor-<br>izin  | Phlor-<br>etin  | <i>p</i> -Hy-<br>droxy-<br>hydro-<br>cinnamic<br>acid | Phloro-<br>glucinol | <i>p</i> -Hy-<br>droxy-<br>benzoic<br>acid | Quercitrin     | Quercetin       |
|-------------------------------------------------------------|-----------------|-----------------|-------------------------------------------------------|---------------------|--------------------------------------------|----------------|-----------------|
| Rf values                                                   |                 |                 |                                                       |                     |                                            |                |                 |
| A                                                           | 0.56            | 0.28            | 0.51                                                  | 0.65                | 0.70                                       | 0.44           | 0.03            |
| B                                                           | 0.21            | 0.02            | 0.78                                                  | 0.47                | 0.68                                       | 0.09           | 0.00            |
| C                                                           | 0.56            | 0.28            | 0.79                                                  | 0.66                | 0.70                                       | 0.45           | 0.04            |
| D                                                           | 0.38            | 0.10            | 0.77                                                  | 0.60                | 0.63                                       | 0.30           | 0.01            |
| E                                                           | 0.06            | —               | 0.19                                                  | —                   | 0.08                                       | —              | —               |
| Reactions for detection                                     |                 |                 |                                                       |                     |                                            |                |                 |
| DSA**                                                       | Yellow          | Yellow          | Red                                                   | Yellow              | Yellow                                     | Pale<br>yellow | Green<br>yellow |
| DNA***                                                      | Yellow<br>brown | Yellow<br>brown | Dark<br>violet                                        | Yellow<br>brown     | Red                                        | —              | —               |
| Na <sub>2</sub> CO <sub>3</sub>                             | —               | —               | —                                                     | —                   | —                                          | Yellow         | Green<br>yellow |
| Ultraviolet<br>light $\lambda = 258$                        | —               | —               | —                                                     | Light<br>blue       | Dark<br>violet                             | —              | —               |
| AlCl <sub>3</sub> daylight                                  | —               | —               | —                                                     | —                   | —                                          | Pale<br>yellow | Yellow<br>green |
| AlCl <sub>3</sub> Ultra-<br>violet light<br>$\lambda = 366$ | Light<br>blue   | White<br>blue   | —                                                     | —                   | —                                          | Yellow         | Green<br>yellow |

\* Solution A, acetic acid—water (15:85); B, 20 per cent potassium chloride; C, acetone—formic acid—water (5:15:80); D, butanol—acetic acid—water (3:2:95); E, butanol—ammonia (butanol saturated with 1.5*N* NH<sub>3</sub>).

\*\* DSA = diazotized sulfanilic acid.

\*\*\* DNA = diazotized nitraniline.

The size of spots on the paper and the intensity of the color reaction indicated that the concentration of phlorizin is less in the woody part than in the bark, while the reverse is true of quercitrin.

A comparison of the Rf values and the color reactions of the identified compounds phlorizin and quercitrin with the substances formed in water culture and water leached through soils containing apple residues, has shown that phlorizin and substance I are identical in all details. It was not possible, however, to identify quercitrin as any of the other substances II to V.

Of the 5 phenolic substances occurring in water culture and leaching water only phlorizin is a natural constituent of the apple roots. It is to be

supposed, therefore, that the other compounds are microbial transformation or decomposition products of phlorizin. If this assumption is true, it should be possible to detect the unidentified components II to V in soils, which contain the phlorizin.

#### DECOMPOSITION OF PHLORIZIN IN SOIL

For the detection of the breakdown products of phlorizin, 1 or 2 g. of the pure chemical were added to 100 g. of soil. The soil was held at a temperature of 22° C. Every 24 hours, and later every 2 to 3 days, 10 g. of the soil were extracted with 50 ml. of ethanol for one hour in a Soxhlet apparatus. The extract was evaporated to 10 ml. and kept 24 hours in a refrigerator. After filtration the extract was ready for the paper chromatographic separation. The experiments were repeated three times.

In three soils from different locations four phenolic compounds in addition to the phlorizin added could be detected with the same Rf values and color reactions as the substances found in water culture and water leaching through soils which contained apple root residues (Fig. 3). Apparently, then, the compounds II to V are decomposition products of phlorizin. The sequence of the appearance of the different compounds is quite similar to the results recorded in Table III. Substance II was detectable in ethanol extracts of the soil after 1 to 2 days, III and IV after 2 to 6 days, and V after 6 to 10 days. These statements refer to soil with a water capacity of 28.5 per cent. The time at which the compounds appeared depended on the experimental conditions, but the sequence of the appearance was the same in all experiments.

The decomposition of phlorizin is accomplished by the soil microorganisms, since no detectable breakdown was found in sterilized soil.

#### IDENTIFICATION OF THE DECOMPOSITION PRODUCTS OF PHLORIZIN

It has been shown above that phlorizin is liberated from apple root residues in soil and nutrient solution and is broken down there into four detectable phenolic compounds. In further experiments it was possible to identify these compounds as phloretin (II); *p*-hydroxyhydrocinnamic acid (III); phloroglucinol (IV); and *p*-hydroxybenzoic acid (V) (3). The different compounds were identified by comparing the Rf values and color reactions of the detected substances (I to V) with the pure chemicals. All the Rf values and color reactions are entered in Table IV. The pure phlorizin, phloroglucinol and *p*-hydroxybenzoic acid used were obtained from Amend Drug & Chemical Co., Inc., New York, and Eastman Kodak Co., Rochester 3, N. Y.; phloretin was produced by hydrolyzing phlorizin (28); and *p*-hydroxyhydrocinnamic acid was obtained by synthesizing *p*-hydroxycinnamic acid (25) and hydrogenating the double bond (12). Acid solutions were used for the paper chromatographic separation of these substances since alkaline solutions resulted in very low Rf values and a



FIGURE 3. Paper chromatographic separation of materials in a soil extract, 7 days after an addition of pure phlorizin. Solution: Ac—H<sub>2</sub>O (15:85 v/v); color reaction with diazotized sulfanilic acid. From left to right: phloretin, *p*-hydroxybenzoic acid, phloroglucinol, soil extract, phlorizin and *p*-hydroxyhydrocinnamic acid.

conversion of phloroglucinol and phloretin (21, 29). The *R<sub>f</sub>* values and the color reaction of the pure substances corresponded to the detected compounds in water culture (I to V), in water leached through soil with an addition of apple roots (I to IV), in root extracts (I), and soil extracts with an addition of pure phlorizin (I to V).

The appearance of the phenolic substances in soil and water cultures





As the previous experiments have shown, a decomposition of the detected compounds also occurs in water culture. The nutrient solution was, therefore, changed at three weeks in order to keep the concentration of test substance at a more constant level. After 12 weeks, the experiments were evaluated as described above. These tests were continued for 84 days as compared to 33 days for the water cultures previously described since a longer time was required for typical "soil sickness" symptoms to appear.

The results of these experiments are presented in Figure 4. The differences of the growth are specified in per cent of the control (= 100 per cent). Only those values deviating from the control values and not ranging within the standard deviation are given in Figure 4. When the data were taken the control plants had an average stem length of 40 cm. and a root length of about 23 cm.

The effect of the compounds on the growth of apple seedlings was not uniform. The results indicated an increasing inhibition effect in the following sequence: phloroglucinol > *p*-hydroxyhydrocinnamic acid > *p*-hydroxybenzoic acid > phloretin > phlorizin. The influence on the root system was most apparent, while the length of stem was inhibited mainly by phloretin and phlorizin (Fig. 5). An average of the five tested substances gave the following sequence with increasing inhibition effect: number of leaves > dry weight of stem > length of stem > dry weight of leaves > dry weight of roots > length of roots.

Similar results with chemicals of the same or similar structure have already been obtained with cereals and flax (1, 2, 4). It seems, therefore, that the phenolic compounds in general have an influence on root growth.

Also the pure substances may be broken down even in nutrient solution. In order to examine this question, every three weeks a portion of 2000 ml. nutrient solution from the water cultures was evaporated to 100 ml. at a temperature of 80° C. After cooling, 200 ml. of ethanol were added. The precipitate was then filtered off and the solution evaporated further to 25 ml. The alcoholic solution (20 to 40  $\mu$ l.) was used for paper chromatography.

Paper chromatograms revealed that, after three weeks of water culture, phloroglucinol and *p*-hydroxybenzoic acid were no longer detectable and *p*-hydroxyhydrocinnamic acid occurred only in traces. Likewise, phloretin and phlorizin were not detectable, but the breakdown products, *p*-hydroxyhydrocinnamic acid and phloroglucinol, could be identified. These facts must be taken into consideration in a critical examination of the effectiveness of the tested substances. Only at the beginning of a growth period of three weeks were the added concentrations ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}M$ ) of the compounds present.

Of the tested compounds, phloretin and phlorizin had the highest

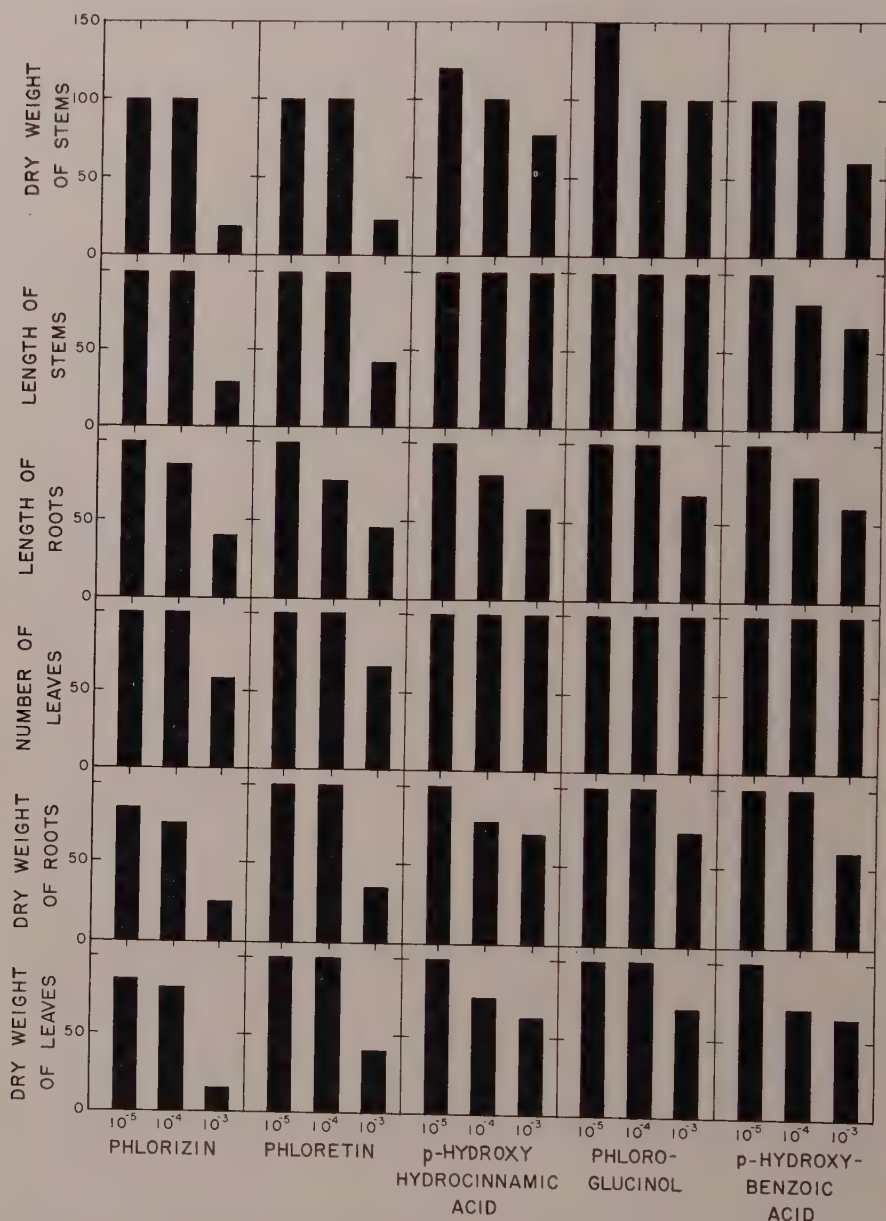


FIGURE 4. The influence of phlorizin and its decomposition products on the growth of apple seedlings in water culture. Duration of experiment 84 days. Inhibition or promotion given as per cent of control.

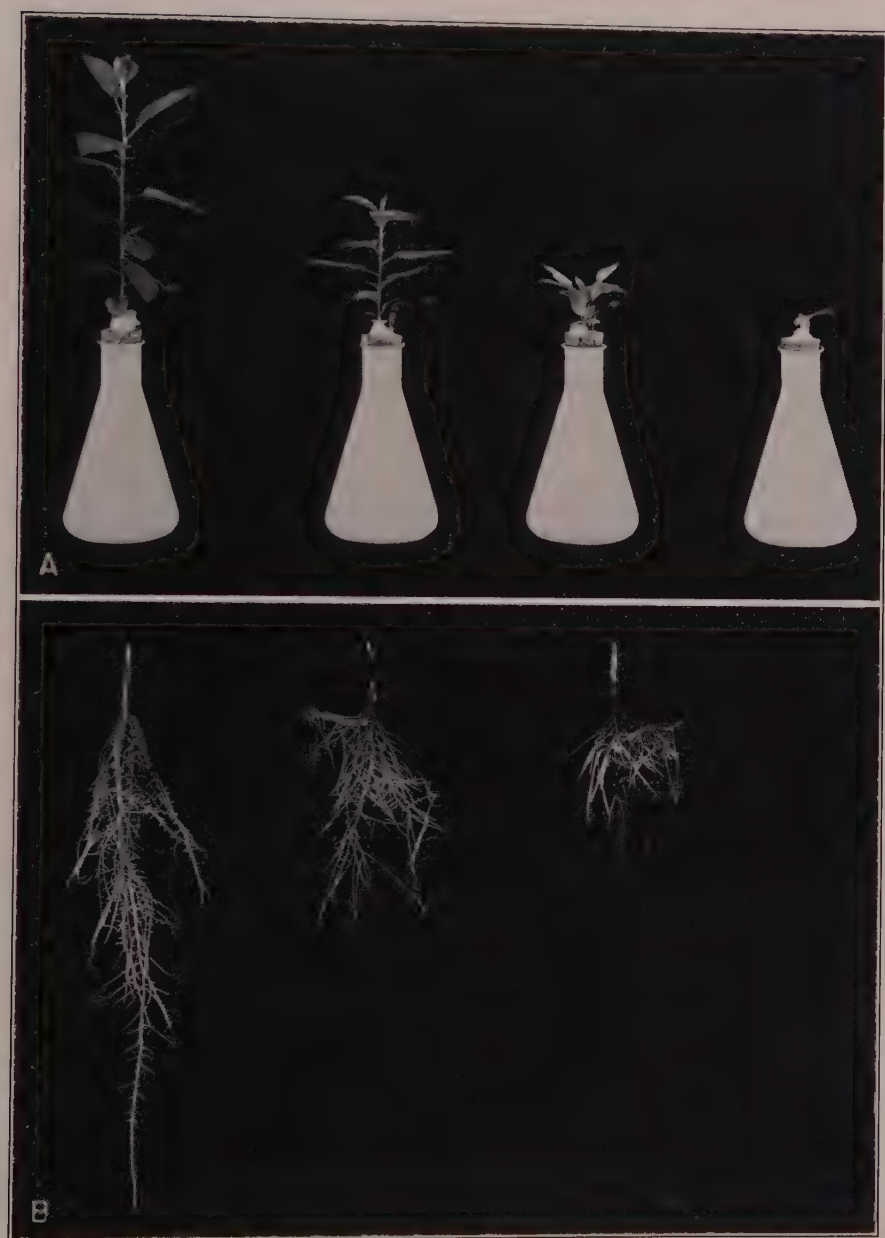


FIGURE 5. Influence of phlorizin on the growth of apple seedlings in water culture. Concentration from left to right: control,  $10^{-5}$ ,  $10^{-4}$ ,  $10^{-3}M$ . Photographed after a culture duration of 84 days. A. Seedlings in culture. B. Corresponding excised roots.



effectiveness. It should be noted, however, that the decomposition products *p*-hydroxyhydrocinnamic acid, phloroglucinol and *p*-hydroxybenzoic acid, are present in the nutrient solution at the same time. Thus it is not clear whether phlorizin or phloretin or the breakdown products are more effective in inhibiting growth.

The inhibiting effect in the experiments with apple roots is more evident than in the experiments with the pure phlorizin. Apart from the different duration of the experiments it should be pointed out that there is an average content of 3 to 5 per cent phlorizin in the root bark (31, p. 442), therefore, at least 6, 30 or 300 mg. phlorizin may diffuse into the solution from the 0.2, 1 and 10 g. of bark used. In the experiments with the pure chemical, on the other hand, 2.16 ( $10^{-5}M$ ), 21.6 ( $10^{-4}M$ ), and 216 mg. ( $10^{-3}M$ ) were present in the cultures. Also, phlorizin diffused very slowly but continually out of the bark, so that there was a constant supply of phlorizin as well as its decomposition products in the solution. This accounts for the fact that, even after five weeks in the flasks containing 10 g. of bark, phlorizin and its breakdown products are detectable in a fairly high concentration, while in the flasks with an addition of pure phlorizin only the compounds phloroglucinol and *p*-hydroxyhydrocinnamic acid could be detected after three weeks. The possibility of the diffusion of other, as yet undetected, substances into the nutrient solution and the effect of the microorganisms themselves should not be overlooked.

### CONCLUSIONS

The excretion of phlorizin from apple roots raises the question of whether or not this compound, as well as the breakdown products, phloretin, phloroglucinol, *p*-hydroxyhydrocinnamic acid and *p*-hydroxybenzoic acid, are of importance in the apple "soil sickness" problem. The answer depends on three factors: a) the physiological effectiveness of the identified compounds, b) their concentration in the soil, and c) the ability of the microorganisms to decompose these substances.

Water culture experiments have shown that the growth of roots and the dry weight of leaves of apple seedlings are inhibited by several of these compounds even at a concentration of  $10^{-4}M$ ; however, the compounds are present in the stated concentration only for a very short time since they are decomposed more or less rapidly.

Whether or not the identified compounds have an effect under natural conditions cannot be answered at this time. Further work is necessary to investigate the quantities of phlorizin which are released from the apple root residues into the soil and the concentration of its decomposition products under natural conditions. Microorganisms undoubtedly play an indirect and possibly a direct role (32). There is the possibility of an activation of the microorganisms which are able to use phlorizin as a carbon

source. Also this substance or its breakdown products may have anti-microbial properties.

MacDonald and Bishop (17) have shown that phloretin, even at a concentration of 30 p.p.m., inhibits the development of gram-positive bacteria while *Aspergillus niger* and *Penicillium* sp. are not influenced. Whether a disturbed microbial balance in soil has an influence upon higher plants is not quite clear at this time. The observation that "soil sickness" can be removed by steaming or treatment with chemicals, such as CS<sub>2</sub> or chloropicrin (9, 27), is strong evidence that microorganisms have a role in the apple replant problem.

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# EFFECT OF TEMPERATURE, LIGHT AND GIBBERELLIC ACID ON STEM ELONGATION AND LEAF DEVELOPMENT IN PHYSIOLOGICALLY DWARFED SEEDLINGS OF PEACH AND RHODOTYPOS

FLORENCE FLEMION

## SUMMARY

The development of normal stems on seedlings from unchilled seeds of peach [*Prunus persica* (L.) Stokes] occurred frequently but not invariably when held under either constant illumination (4000 to 5800 Å) or when daylight was supplemented at night with intermittent light. By filtering the supplementary light, tall spindly stems and thin pale green leaves were produced by infrared radiation of wavelengths longer than 6950 Å while shorter sturdier stems and thick dark green leaves developed when the filter transmitted wavelengths between 5800 and 6500 Å. Regardless of the green coloration or texture of the foliage or whether or not the internodes had been extended, seedlings from unchilled peach seeds when held under these various light conditions produced leaves which were sometimes misshapen and which resembled the malformed leaves of the physiologically dwarfed seedlings from unchilled seeds grown under daylight conditions in the greenhouse.

With either short photoperiods (2 hours of daylight and 22 hours of darkness) or application of gibberellic acid in lanolin, the internodes of actively growing peach seedlings from unchilled seeds were stretched so rapidly that extremely spindly stems were obtained. Successive treatments with gibberellic acid were required in order to prevent the seedlings from reverting to the telescoped internode condition of the dwarfs. When gibberellic acid treatment was combined with supplementary light conditions, the etiolation effects were gradually overcome and eventually there were no obvious differences between treated and untreated seedlings of peach and *Rhodotypos kerrioides* Sieb. & Zucc. Soaking either unchilled or partially-chilled peach seeds in various concentrations of gibberellic acid had no effect on subsequent development of the seedlings.

The apical buds of dormant peach seedlings often resumed growth without chilling when daylight was combined with intermittent light at night; but when the dormant tips were held in constant darkness, apical growth did not occur. Exclusion of light from the tips of actively growing seedlings from either chilled or unchilled seeds produced extreme etiolation. Subsequently, when the apices were exposed to daylight, no further elongation occurred; however, whorls of leaves developed at the tips. This pattern of arrested development is characteristic of the physiologically dwarfed condition.

The chilling requirements of seeds of peach and *Rhodotypos* could, to some extent, be dispensed with by combining daylight with intermittent supplementary light at night, and, although the internodes could be elongated in this manner and appeared normal, basic processes were apparently not functioning properly since some of the leaves on the main axis of the plants were distorted and malformed.

## INTRODUCTION

Seeds of peach [*Prunus persica* (L.) Stokes] and *Rhodotypos kerrioides* Sieb. & Zucc. were used to study the effect of temperature, light and gib-

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berellic acid on dormancy and growth because seedlings could be obtained without the prechilling treatment (4, 7, 8, 18). Since the unchilled seeds produced physiological dwarfs, responses such as overcoming the lack of elongation of the internodes and or the elimination of abnormal leaves could be readily observed in the various experiments.

Light effects were investigated for when the apex of a dwarfed seedling of peach or *Rhodotypos* occasionally developed normally it occurred usually, but not invariably, in the springtime (7, 8). In addition, the dwarfed seedlings were extremely stunted when grown under winter daylight conditions in the greenhouse, whereas when planted during the longer days of spring, the internodes tended to be less telescoped which was, however, associated with a greater tendency for the development of distorted leaves. In view of these observations and of favorable light effects on peach (13, 17) and on buds of various woody species (6, 11, 12, 19 and others) a number of different conditions were investigated. Since gibberellic acid had been used to stretch normal stems of apple, rose etc. (15) and had been suggested as a possible substitute for the cold requirements of some plants (14) and seeds (2, 3, 5, 10), both dwarfed seedlings and seeds were treated with this material, then grown under conditions in the greenhouse with and without supplementary light.

The results of these various studies on stem elongation and leaf development are presented below.

#### MATERIALS AND METHODS

The 1953 and 1956 crops of peach seeds obtained from a propagated, selected, wild strain grown in Tennessee and known as "Superior Peach Pits" were stored dry in closed containers at 5° C. while the *Rhodotypos* seeds were collected on the Institute grounds and held at room temperature. For the production of seedlings from chilled and unchilled seeds, techniques previously described (7, 8) were employed, and all of the plants, unless otherwise indicated, were grown in soil in four-inch clay pots in the greenhouse which was maintained, except during the warmer months, at approximately 22° C. (72° F.). To prevent the soil from drying out too rapidly, especially in the various light experiments, 12 pots were embedded in a greenhouse flat (20 inches×15 inches×3 inches) with granulated peat moss which was kept moist.

For continuous illumination a temperature-regulated light room was maintained at approximately 24° C. (75° F.) during the day and 18.5° C. (66° F.) at night. The light source was AH mercury arc lamps which delivered 2500 to 2000 foot candles at a distance of 3 feet from the floor. The relative energy of this light was mainly in the blue-violet and green-yellow portions of the spectrum (4000 to 5800 Å).

For supplementary light in the greenhouse, General Electric incandes-

cent filament lamps were used and, in order to avoid excessive heat from these lamps, the lights were intermittently controlled. At the time of planting the height of the 500-watt lamps was adjusted in order to maintain approximately 1000 foot candles of light at the soil level, and, as the plants developed, the lamps were raised to maintain a distance of about 18 inches above the growing tips. When the intermittency of light was controlled by a soil-heating cable thermostat (1), it was adjusted to deliver sufficient lighting periods to maintain a temperature of 19° to 23° C. (67° to 73° F.) and was suspended directly beneath and approximately 8 inches below one of the lamps operating on this circuit. Since this mechanism operated on a 24-hour basis, the light was on frequently whenever the temperature dropped to 19° C. Subsequently, the circuit was cut off when the thermostat which was covered with black tape to quickly absorb the heat from the lamps registered 23° C. The total amount of time the lights were on in each 24-hour period was recorded by an electric clock connected to the circuit. The other intermittency light, not temperature controlled, was obtained with a powerstat variable transformer set to deliver from 4:30 P.M. to 8:30 A.M., constant 3-minute intervals of light with constant 3-minute intervals of darkness. The lights during this 16-hour cycle were on for a total of approximately 8 hours. The cable thermostat which varied according to the greenhouse temperature averaged (during a 150-day period from December 23, 1955 to May 21, 1956) approximately 11 hours of lighting for each 24-hour period.

When filtering the radiation from either sunlight or the lamps, transparent colored DuPont cellophane was used. For transmission beyond 6950 Å (16, p. 468) a 36-inch square wooden frame covered with two layers of red plus two layers of dark blue cellophane was placed over the seedlings. When screening only the supplementary light, a small wooden frame covered as above enclosed the 100-watt incandescent lamp on all sides except for the top where the lamp shade was located. For red radiation between 5800 to 6500 Å a Westinghouse fluorescent daylight lamp (40 watts) was covered with two layers of the red cellophane. A General Electric radiation meter was used periodically to measure the light intensity (heat value), and, as the seedlings developed, each lamp was raised in order to maintain approximate readings at the apices. At about the same distance above the tips of the plants the 60-watt lamp without filters and the 100-watt lamp with filters which transmitted only the far and infrared radiations each delivered approximately 0.005 to 0.010 g. calories per sq. cm. per minute. For a comparable value the fluorescent lamp, which was filtered and transmitted only the red region of the spectrum, had to be placed very near the tips of the growing seedlings. While each lamp was in a separate compartment with flexboard partitions, all three were on the same electrical circuit so that the intermittency of light

TABLE I  
SPECTRAL LIMITS IN THE VISIBLE REGION AND TRANSMISSION OF RADIANT ENERGY  
OF SUNLIGHT IN DIFFERENT GREENHOUSES

| House number | Type of glass       | Visible and ultraviolet range—Angstrom units (Å) | Approximate solar energy transmission, per cent |
|--------------|---------------------|--------------------------------------------------|-------------------------------------------------|
| 1            | Alko heat absorbing | 4000–7200                                        | 55                                              |
| 2            | Ordinary greenhouse | 3120–7200                                        | 89                                              |
| 3            | G403E.D.            | 3840–5850                                        | 22                                              |
| 4            | Noviol "C"          | 4720–7200                                        | 55                                              |
| 5            | G 34                | 5290–7200                                        | 54                                              |

was identical. In addition, five spectral greenhouses were used which also screened out various wavelengths of sunlight. Table I, which was prepared by Dr. John M. Arthur, lists the different kinds of glass in these houses and the regions of the spectrum which each transmitted.

When the daylight hours in the greenhouse were reduced the plants were covered daily for specific time intervals with light-proof boxes covered on the outside with aluminum foil and on the inside with black paper. When light was excluded from only the growing tips, the seedlings were capped with several layers of black silk and/or aluminum foil which was held firmly to the stem with very thin wire. As subsequent development occurred, the cap was adjusted and raised (without exposing the apex to light) every day or two in order to avoid crowding of the actively growing tip under the cap.

Samples of gibberellic acid were obtained through the courtesy of Dr. A. J. Vlitos who had obtained "Gibberellin" from Imperial Chemical Pharmaceuticals of Manchester, England, Dr. P. W. Zimmerman who had obtained "Gibberellin B and Gibberellic NORB" from United States Department of Agriculture, and Dr. Reed A. Gray of the Microbiology Department, Research Laboratories, Merck, Sharp and Dohme, Rahway, New Jersey, who sent a sample of Gibrel—a potassium salt of gibberellic acid. The material was either mixed in lanolin (10 mg. per g. of lanolin), and some of this preparation was applied to the stem, leaflets, leaves or cotyledons or placed in solution (10 p.p.m.), drops of which were applied directly on the seedling apex. In one test a total of 30 drops was applied over a two-week period while in another a total of 27 drops was used during an interval of four weeks. Tergitol was used in these liquid applications. In both the lanolin and liquid treatments the exact amount of gibberellin applied and the quantity taken up by the various tissues were unknown. When seeds were treated (with and without inner coats) they were soaked (60 seeds per 60 ml.) for 24 hours at room temperature in concentrations of 0.20, 100 and 200 p.p.m. Those soaked with inner coats intact

were either planted directly in the soil or the embryos were excised and planted after germination had taken place on moist filter paper in Petri dishes at room temperature.

## RESULTS

### TEMPERATURE EFFECTS

Physiologically dwarfed peach seedlings—characterized by telescoped internodes of the stem—when held in a warm greenhouse did not usually resume growth and while they appeared to be in a rather quiescent condition, they gradually increased in girth. When growth, on occasion, occurred, it was frequently a branch which was produced and, as shown in Figure 1 which illustrates a physiological dwarf held constantly for ten years at a warm temperature, these branches also often lacked the ability to elongate. However, when branches developed during the initial growth of the main axis of these seedlings from unchilled seeds they were always normal. Of the large number of dwarfed peach seedlings used in this laboratory throughout the years, there were only two instances when the leaves on these branches were not also normal. On the other hand, leaves



FIGURE 1. Development of physiologically dwarfed peach seedling held for 10 years in a warm greenhouse. Natural size.



which developed on the main axis were often distorted and while they not only varied from one to many on a given seedling, there was no particular pattern as to their order of appearance. It has long been known that normal stems and leaves are produced by adequately chilling either the seeds or the dormant vegetative buds of various species of plants from the temperate zone regions. Likewise, physiologically dwarfed seedlings of various ages also responded to chilling and after 6 to 9 weeks at 5° C. normal development invariably occurred.

#### LIGHT EFFECTS

*Constant light room.* Dormant physiologically dwarfed seedlings of peach (Fig. 2 B, left) when transferred in December from the warm greenhouse and held under constant illumination for 47 days produced mostly



FIGURE 2. Development of peach seedlings from unchilled peach seeds (A and B,  $\times 0.15$ ; C  $\times 0.44$ ). A. Planted December 1954 and held for 7 weeks in the greenhouse with daily exposure to sunlight for (left to right) normal day length, 6 hours, and 2 hours. B. Planted March 1954 and photographed 12 months later. Greenhouse control (left). After 9 months in greenhouse transferred to 5° C. (center) or to constant light room (right) for 47 days then returned to greenhouse for 45 days. C. Planted and held in constant light room for 38 days.

branches as shown in Figure 2 B, right. No additional growth occurred when the plants were returned to the greenhouse. On the other hand, seedlings from the same series when held for the same length of time at 5° C. instead of in the constant light room subsequently produced, when transferred to the original greenhouse, normal stems and leaves (Fig. 2 B, center). In another experiment unchilled peach seeds at the time of planting were placed in the constant light room. In approximately 75 per cent of the seedlings produced under these conditions, the stems failed to elongate; however, each plant with telescoped internodes had developed one or several branches which were normal in every respect. In the other 25 per cent although the stems had elongated and appeared normal the leaves on some of the plants were abnormal. An extreme example is shown in Figure 2 C. No further growth occurred in any of the seedlings when transferred on the 38th day to the warm greenhouse.

*Daylight supplemented with intermittent light.* Germinated unchilled peach seeds were placed immediately after planting in December under the lights controlled by the cable thermostat while others which served as controls were kept in another section of the same greenhouse where daylight was the only light source. Since the lights were controlled by the temperature in the greenhouse, they were on frequently during the night and sometimes during the day. Within six weeks the controls had developed telescoped internodes and were approximately 49 mm. tall while the seedlings with supplementary light had attained an average height of 178 mm. These elongated seedlings with leaves which were sometimes misshapen resembled those obtained in the constant light room. On March 1 all of the plants under the lights were transferred to another section in the greenhouse where the controls were kept and were henceforth subjected only to daylight where very little subsequent development occurred.

Another similar experiment was started in February and differed only from the above in that the supplementary light at night was controlled by a variable transformer. Under these conditions of daylight combined with 16-hour intermittent light approximately 50 per cent of the seedlings which were produced had telescoped internodes with normal branches. In the others, the stems had elongated to an average height of 250 mm. and in some cases there was an abnormal thickening of the stem in the region of the apex. In many of these plants, whether the main stem had elongated or remained dwarfed, distorted leaves were frequently present on the main axis. Peach seedlings from adequately chilled seeds when held from the time of planting under these conditions were normal in every respect except that they were taller than the controls. When germinated unchilled *Rhodotypos* seeds were planted in January in the greenhouse, the seedlings produced under the prevailing daylight conditions were extremely stunted and had small dark green leaves (Fig. 4 A, 1st left) while those

which had developed when daylight was supplemented with 16-hour intermittent light at night had elongated stems (Fig. 4 A, 2nd right). Subsequently when all development had ceased, the control seedlings had remained dwarfed (approximately 60 mm. tall) while the average height of those under supplementary light was approximately 343 mm. and, in about 25 per cent of the plants, the leaves had remained small to medium.

*Filtered radiation.* A cage in the greenhouse which screened both daylight and the 16-hour intermittent supplementary light (transmission beyond 6950 Å) was placed over germinated unchilled peach seeds when planted in January 1957. The extremely etiolated seedlings were removed 17 days later and the only subsequent development which occurred in those which were thereafter subjected to daylight was a whorl of leaves at the tip of each. In the others when daylight was supplemented with light at night apical development that appeared to be normal occurred within four weeks, but the original spindly regions of the stems had remained practically unchanged although the tiny leaves had enlarged slightly.

Another experiment differed from the above in that only the supplementary light was filtered. Under this set of conditions the red radiation (5800 to 6300 Å) produced short, stocky stems and large, thick, dark green leaves, the far red and infrared (beyond 6950 Å) produced thin, light green leaves and stems that were considerably taller which tended to be spindly, while intermediate development was obtained when the light from the incandescent lamp (3200 to 30,000 Å) was not filtered. Although daylight when thus combined with these different wavelengths of intermittent light at night produced such marked differences in the development of seedlings from unchilled peach seeds, the occurrence of malformed leaves persisted. These light conditions also had some effect on the development of seedlings from chilled peach seeds for although the heights were all about the same the stems produced with red radiation were very sturdy, those with infrared tended to be spindly, while those with unfiltered radiation at night were intermediate and appeared normal. The leaves in these various seedlings were also normal except for the darker green color produced by the red radiation at night.

Several years prior to the above, seedlings from unchilled peach seeds which had been planted in October were held until January in five separate spectral houses. Thin elongated seedlings were produced when all wavelengths shorter than 4720 Å were eliminated, while dwarfing (lack of stem elongation) became more pronounced as transmission of the shorter wavelengths increased down to 3120 Å. Upon transfer of these various seedlings to an ordinary greenhouse no further development occurred except for a whorl of leaves which developed at the apex. A similar experiment was conducted during the spring and early summer with peach seedlings from both chilled and unchilled seeds. Although the heat on



sunny days was not very favorable for the growth of peach seedlings, the plants were taller than when grown in the late fall as in the previous experiment. While many of the seedlings from unchilled seeds appeared normal in respect to height and size of stem, many of the leaves were malformed.

*Short photoperiods.* When the daily exposure to sunlight in the regular greenhouse during the winter was considerably reduced, extensive stretching of the internodes occurred in seedlings from unchilled seeds. Six hours of daylight or the regular day length prevailing at the time produced dwarfs (Fig. 2 A, left and center) while a daily two-hour exposure to daylight combined with 22 hours of darkness produced etiolated plants (Fig. 2 A, right) with very pale green leaves. The size and shape of these leaves were about the same as those on the dwarfed seedlings.

*Light excluded from apex.* The shoots of peach seedlings when visible above the soil (December 1954) were covered and held in constant darkness for various periods of time in the greenhouse. Under these conditions etiolation occurred and subsequently when the tips were also exposed to daylight the seedlings ceased to elongate, the spindly stems gradually increased in girth, the tiny leaves enlarged, a whorl of leaves developed at the apex, and sometimes clusters of leaves were produced along the stem. The photographs in Figure 3 of seedlings from both chilled and unchilled seeds capped for various periods of time illustrate the profound changes which occurred in both types of seedlings. These modifications were also obtained in other experiments when light was excluded for shorter and longer periods of time (14 to 93 days). Seedlings from chilled seeds appeared to have a tendency to produce clusters of leaves and branches near the base (Fig. 3 B, second from left). Instead of maintaining the apex in constant darkness from the time of planting a number of seedlings from chilled seeds were permitted to develop in the greenhouse to various heights (40 to 170 mm.) before excluding light from the actively growing tips. Here, also, the new growth of the main axis which occurred during the period that the apex was held in darkness was extremely etiolated.

An experiment undertaken in October 1957 with seedlings from unchilled seeds differed from the above in that daylight was supplemented with intermittent light at night during the 21-day period that the growing tips were held in constant darkness. Etiolation also occurred under these conditions and when the caps were removed some of the seedlings remained under the supplementary light while others were transferred to another section in the greenhouse where only daylight was available. In both cases the spindly stems and small occasionally distorted leaves gradually enlarged; however, with supplementary light (whether controlled by thermostat or transformer) resumption of apical development occurred in approximately 30 per cent of the seedlings. This new growth resembled





FIGURE 3. Effect of excluding light from the apices of actively growing peach seedlings. Planted in greenhouse December 1954 and photographed after 50 (A) and 95 (B) days ( $\times 0.17$ ). A. Left to right: control from chilled seed, seedling from chilled seed capped for 40 days, seedling from uncapped chilled seed capped for 50 days, and control from uncapped chilled seed. B. Same plants as in A except photographed 45 days after removal of caps of two center seedlings.

that obtained in approximately 42 per cent of the seedlings which had not been capped but which from the time of planting had been subjected to daylight combined with the supplementary light. The stems appeared normal while some of the leaves were distorted.

The effect of excluding light from the apical buds of dormant seedlings was also investigated. Normal peach seedlings from chilled seeds planted in September 1955 had been dormant in the greenhouse for several months when 22—selected in February for uniformity (no branches; each 340 mm. tall)—were subjected to various conditions. When the terminal buds and stems were exposed to daylight supplemented with the 16-hour intermittent light at night, within a month all produced normal apical growth when the leaves were either absent, removed, or covered with aluminum foil, while only some of the apices developed when the leaves had been retained. There was no new growth of the terminal buds when they had been kept in constant darkness by capping irrespective of supplementary light or of the presence or absence of old leaves; however, on the stems where old leaves had been removed, clusters of small leaves often developed.

#### GIBBERELLIN EFFECTS

*Peach.* A single application of the lanolin preparation when placed on the stem near the actively growing tip did not have much effect on elongation; however, considerable etiolation was obtained when three successive applications were spaced from four to six days apart. Of the fifteen seedlings from unchilled seeds treated in this manner (January 1957), the five shown in Figure 4 B (second from left up to and including second from right) illustrate the two extremes as well as some of the typical intermediates. In addition to considerable variation in stem development many of the leaves were abnormal as to size, shape and color (very pale green). Sturdy seedlings with normal medium green leaves were obtained by planting chilled seeds (Fig. 4 B, extreme right). Gibberellic acid in lanolin was also applied to the tiny leaflets and cotyledons of 36 unchilled peach seeds when planted on March 11 and again, ten days later, to the stem near the tip. The differences between treated and nontreated (36 controls) seedlings when held for two months under daylight conditions were similar to those just described and shown in Figure 4 B. With supplementary light at night the spindly etiolated stems gradually became quite sturdy and although eventually both the treated and nontreated seedlings had what appeared to be normal stems the leaves were often malformed. Seedlings from unchilled seeds with three applications (1st, 3rd and 11th day) of the lanolin preparation were grown in liquid nutrient culture under daylight conditions in the greenhouse with and without supplementary light. Here, also, the differences were striking at first but eventually there



FIGURE 4. Effect of gibberellin on growth of seedlings from unchilled seeds ( $\times 0.08$ ). A. *Rhodotypos* seedlings photographed 45 days after planting. Two plants on left received prevailing sunlight in the greenhouse while the two on right received in addition intermittent light at night. In each pair untreated control on left, and on right gibberellin in lanolin was applied to the stem on the 4th day after planting. B. Peach seedlings photographed 66 days after planting in the greenhouse. Extreme left, untreated control. Second left up to and including second on right, three applications of gibberellin in lanolin applied to each stem on the 7th, 13th and 17th day after planting. Extreme right, normal seedling (seed chilled for 92 days at  $5^{\circ}\text{C}$ .).

were no obvious differences between the treated and control plants.

Prior to the above treatments two experiments had been conducted in which gibberellic acid in solution was applied directly to the actively growing tips of seedlings from unchilled seeds but these treatments were ineffectual. Various solutions were also used for seed treatments. In December 1957 immediately after partially-chilled seeds (29 days at  $5^{\circ}\text{C}$ .) with inner coats intact had been soaked, 30 from each concentration were planted in the greenhouse while the other 30 seeds from each treatment were excised and germinated prior to planting. Unchilled seeds were also treated and handled in a similar manner. When seeds, whether treated, nontreated, unchilled, or partially-chilled, were planted with inner coats intact only a few seedlings were obtained whereas approximately 90 per cent of the germinated excised embryos developed into seedlings when placed in soil. Gibberellic acid in these tests had no effect since seedlings from unchilled seeds were dwarfed and those from partially-chilled seeds were semi-dwarfed.

*Rhodotypos*. The gibberellic acid lanolin preparation was applied in

January 1957 to the tiny leaflets and cotyledons of seedlings from unchilled seeds on the fourth day after planting. Within a few days there was extensive stretching of the internodes while the small leaves were very pale green in color. Two months after the photographs were taken which illustrate this response in Figure 4 A (second from left and extreme right), the treated seedlings held under daylight conditions had remained spindly and averaged 140 mm. in height, while those where daylight had been supplemented at night with intermittent light had an average height of 295 mm. In addition, the etiolated regions of the stems had become sturdier and the leaves had acquired a darker green color. Similarly, the seedlings without gibberellic acid treatment had remained physiologically dwarfed under daylight conditions, while with supplementary light they were sturdy, appeared normal, and had an average height of 345 mm.

#### DISCUSSION

In the present studies supplementary light served to some extent as a substitute for the chilling requirements of peach seeds by overcoming the inability of physiological dwarfs to elongate; however, in these seedlings the shoot apex, where the seat of dwarfishness is located (9), continued to manifest the lack of chilling by retaining the tendency to produce misshapen leaves on the main axis. It has been suggested that light absorption for stem elongation differs from that for leaf development (20) and that the site of photoreception in relation to dormancy is located in the meristematic tissues of the bud (19). Seedlings of peach and *Rhodotypos* from chilled seeds were in some manner either less demanding or perhaps more responsive to light since normal leaves and stems were always produced by the daylight prevailing in the greenhouse at any time of the year including the short days of winter; whereas, these conditions were inadequate for seedlings from unchilled seeds. Various investigators have shown that many changes occur during the chilling of seeds and vegetative buds but what is not known is the chemical environment at the cellular level which influences in some manner the normal development of stems and leaves.

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# DARK CARBON DIOXIDE FIXATION BY UREDOSPORES OF RUST FUNGI<sup>1</sup>

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## SUMMARY

Uredospores of both *Puccinia recondita* Rob. ex Desm. and *Uromyces phaseoli* (Pers.) Wint. fixed carbon dioxide in the dark yielding malic, malonic and aspartic acids as early products. Other intermediates of the Krebs cycle also became labeled during longer periods of fixation, but the importance of the Krebs cycle in endogenous uredospore metabolism is unknown. Evidence is presented to support the hypothesis that oxalacetate is the product of phosphoenolpyruvate carboxylation.

## INTRODUCTION

Uredospores of *Puccinia recondita* Rob. ex Desm. (5) and *P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. possess most of the intermediates of the Krebs cycle (22). Except for this chemical evidence there is little information that the cycle is actually operating in endogenous spore metabolism, since spore respiration is not inhibited by malonate, nor is it responsive to most cycle intermediates except succinate (22).

Dark carbon dioxide fixation has been described in many higher plants (13, 19, 26), fungi (11), and bacteria (1, 24) and the mechanism elucidated in great detail (2, 19, 20, 25, 26). If it could be demonstrated to occur in uredospores, this fixation would serve as a tool for the study of the Krebs cycle in uredospore metabolism, since dark carbon dioxide fixation processes and the Krebs cycle are intimately associated (20).

One of the most abundant acids in uredospores has been found to be malonic acid (22). A study of dark carbon dioxide fixation could help to clarify the role of this acid in uredospore metabolism, since one mechanism for the formation of malonic acid in tissues involves oxalacetic acid (27).

## MATERIALS AND METHODS

*Uredospores.* Uredospores of the bean rust fungus, *Uromyces phaseoli* (Pers.) Wint., were obtained from greenhouse-grown bean plants. Ten seeds of *Phaseolus vulgaris* L. var. Pinto were planted in each of 72 4-inch pots and placed on the greenhouse bench. Ten days after planting, the leaves were heavily inoculated with bean rust uredospores at the rate of about 0.1 mg. uredospores per leaf. This was done by mixing the spores with talc, suspending the spore-talc mixture in water, and spraying the leaves with a DeVilbiss sprayer using an air pressure of 35 lb./p.s.i. After spraying, the plants were placed in a high humidity chamber at 60° F. (12) overnight. At the end of the incubation period the plants were returned

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to the greenhouse. Twelve days after inoculation, the spores were harvested by brushing them into an enamel pan with a camel's-hair brush. Spores were stored in a refrigerator at 4° C. until required, but never for more than 1 week. Spores used had a germination rate of greater than 90 per cent.

Wheat leaf rust uredospores were obtained from field collections as described previously (22).

*Incubation of uredospores with labeled bicarbonate.* Five grams of uredospores were washed on a Buchner funnel with 500 ml. of distilled water, placed in 50 ml. of distilled water, and stirred with 0.1 mc.  $\text{NaHC}^{14}\text{O}_3$  (specific activity = 1.0 millicurie per millimole) in a closed system. At the end of the incubation period the reaction was stopped by pouring the spore mixture into 200 ml. of boiling 30 per cent ethanol. After centrifugation the spores were taken up in 50 ml. of 80 per cent ethanol and ground in a planetary ball mill containing one hundred  $\frac{3}{8}$ -inch porcelain balls until all spores were disintegrated. The supernatant from centrifugation was saved for further analyses.

*Incubation of uredospores with labeled nonvolatile compounds.* Essentially the same procedure as for bicarbonate was followed except that the flask was swept continuously with  $\text{CO}_2$ -free air and radioactive  $\text{CO}_2$  was trapped in  $\text{Ba}(\text{OH})_2$  for counting. For each 5 g. of uredospores, 0.1 mc. of succinic-1- $\text{C}^{14}$  acid (specific activity = 0.75 millicurie per millimole) and 0.025 mc. of fumaric-1- $\text{C}^{14}$  acid were used (specific activity = 0.75 millicurie per millimole).

*Extraction.* The broken uredospores were extracted by stirring with 100 ml. of 80 per cent ethanol. The mixture was centrifuged, ethanol was decanted, and the procedure repeated until the spores had been extracted 5 times. All supernatants were combined.

The uredospores were then extracted with hot water following the method of Palmer (16). After centrifugation the supernatant was decanted, more water added, and the extraction repeated twice more. Supernatants were combined with the ethanol extracts.

*Analysis of intermediates.* Substances in the uredospores were determined after first fractionating the spore extracts into the broad categories of cationic, anionic and nonionic species. This was done by passing the extract through a 1×10 cm. column of hydrogen-form Dowex 50-X4 resin, 200 to 400 mesh, to sorb amino acids. The effluent from this column, containing a mixture of sugars and organic acids, was passed through a 1×15 cm. column of formate-form Dowex 1-X8 resin, 200 to 400 mesh, to sorb organic acids. The effluent, containing mostly carbohydrates, was saved for further analysis.

The free amino acids and amides were eluted from the cation exchange resin by following the method of Plaisted (17). Aliquots of the samples were spotted onto 14×17 inch sheets of Whatman No. 3 filter paper and

were chromatographed in 70 per cent phenol—water (4 parts 88 per cent phenol:1 part water) in the first direction, and in *n*-butanol—acetic acid—water (45:5:14.5 v/v) in the second direction (18).

Separation of the nonvolatile organic acids was accomplished using a modification of the method of Saltman *et al.* (20). The organic acids were eluted *en toto* from the anion exchange resin by addition of 100 ml. of 6*N* formic acid. The formic acid was removed by evaporation in a stream of air and the residue taken up in 1 ml. of distilled water. Aliquots of this acid mixture were spotted onto 14×17 inch sheets of Whatman No. 1 filter paper and chromatographed in 70 per cent phenol—water—formic acid (8 parts 88 per cent phenol:1.9 parts water:0.1 part 88 per cent formic acid) for the first direction. Solvent for the second direction was *n*-butanol—acetic acid—water (65:5:50 v/v). Acids were identified by co-chromatography with standards.

For quantitative analyses of organic acids the gradient elution procedure of Palmer (16) was followed using the modifications previously described (22). Acids were titrated using a phenol red indicator and characterized after isolation by infrared spectrophotometry, melting point, and, where possible, carbon and hydrogen analyses.

Acids isolated for identification were first decolorized by reelution from a Dowex 1 column since the indicator used in their titration, phenol red, is not removed from this resin by formic acid elution. The aqueous solution was then dried in a stream of air, the acid residue taken up in successive portions of anhydrous ethyl ether and dried on a watch glass. The infrared spectra were determined using a Perkin-Elmer Model 13U infrared spectrophotometer by the KBr pellet technique. Acids so identified were also co-chromatographed on Whatman No. 1 filter paper sheets with an authentic sample of the specimen in solvent F of Stark, Goodban and Owens (23). Where sufficient sample was available melting points were determined by standard techniques.

Sugars were identified by a paper chromatographic technique (30). The solutions containing sugars were dried under vacuum and aliquots of the extracts were spotted on 10.5×17 inch sheets of Schleicher and Shuell No. 598 paper along with authentic sugar samples. One-dimensional ascending chromatography was carried out using the upper phase of *n*-butanol—pyridine—water (125:40:125 v/v). Each sheet was chromatographed 3 times. Papers were sprayed with aniline phthalate or naphthoresorcinol and phosphoric acid and developed in an oven at 110° C. for 15 minutes.

Radioactivity was determined on paper chromatograms after the spots were located by radioautography. After chromatography, the filter paper sheets were dried in a stream of air, covered with 14×17 inch sheets of Kodak No-Screen X-ray film, and placed in the dark for up to 1 month. After this time the film was developed and positions of the radioactive



spots on the paper determined. Radioactivity of the spots was measured directly on the paper by an end-window Geiger tube described by Fuller (6). Measurements of radioactivity in amino acids on Whatman No. 3 paper were corrected by multiplying these values by 1.33 to yield values comparable to those obtained on Whatman No. 1 paper.

The "citrate" peak of the gradient elution procedure (16) was found by paper chromatography to consist of citric and malonic acids. To further separate these acids for isolation and quantitative estimation, the silica gel chromatography method of Bulen, Varner and Burrell (3) was followed. Tubes containing the acids of the "citrate" peak were pooled, dried under a stream of air, taken up in silica gel, and the column developed as described (3). The effluent was collected in 2 ml. aliquots on a fraction collector, dried under a stream of air at room temperature and titrated with 0.01*N* NaOH.

Radioactivity in sodium oxalacetate acid was determined by a trapping experiment. Ten milligrams of oxalacetic acid were added to 5 g. of uredospores in 50 ml. water to which 50  $\mu$ c. of  $\text{NaHC}^{14}\text{O}_3$  were added as described earlier. After 1 hour, the mixture was dropped into 50 ml. 0.01*M* 2,4-dinitrophenylhydrazine solution (8) and an additional 100 mg. oxalacetic acid were added. The mixture was agitated for a half hour and the spores were then removed by centrifugation. The hydrazones were recrystallized to constant specific activity and chromatographed using the *tert.* amyl alcohol—ethanol—water solvent (5:1:4 v/v) of Isherwood and Niais (8). Radioactivity was located by autoradiography as before. Infrared spectroscopy was utilized to confirm the tentative identification of the oxalacetic acid hydrazone.

*Malic acid degradation.* Malic acid, isolated from bean rust uredospores incubated with  $\text{NaHC}^{14}\text{O}_3$ , was degraded following the procedure of Nossal (15) for carbon atom 4 and the acid permanganate method of Katz *et al.* (9) for carbon atom 1. Further degradation was not required since the remaining acetate containing carbon atoms 2 and 3 was not radioactive.

## RESULTS

*Bean rust uredospores.* Tables I and II include a list of acids found to be in extracts of bean rust uredospores. Each of the acids was isolated and characterized on the basis of melting point and infrared spectrum. Aconitic acid and the amino acids were identified on the basis of their co-chromatography with authentic standards during filter paper chromatography. Carbon and hydrogen analyses of citric and malonic acids confirmed their analyses by other methods. Thus each of the organic acids found in wheat rust uredospores (22) has also been positively identified to be present in bean rust uredospores. Isocitrate has never been detected in uredospores and in the present experiments it was not demonstrated

TABLE I  
CHANGES IN ORGANIC ACID CONTENT OF BEAN RUST UREDOSPORES DURING STORAGE

| Acid                   | Concentration* of acids after |                |
|------------------------|-------------------------------|----------------|
|                        | 1-Day storage                 | 60-Day storage |
| Pyrrolidone carboxylic | Trace                         | Trace          |
| Succinic               | 70                            | Trace          |
| Malic                  | 47                            | 33             |
| Citric                 | 1863                          | 1702           |
| Malonic                | 822                           | 1081           |
| Fumaric                | 313                           | 52             |
| Aconitic               | 156                           | 113            |

\*  $\mu$ G. per g. fresh weight as determined in duplicate analyses.

either by two-dimensional paper chromatography or by silica gel chromatography, both of which will separate isocitrate from citrate (3, 20).

Most of the acids appear in amounts less than one milligram per gram fresh weight of spores (Tables I and II). Exceptions to this are citric and malonic acids which occur in greater than milligram amounts so that analyses for them are reasonably accurate, and they would seem to be good indicators of metabolism when the spores are subjected to a varying environment. Amino acids derived from the Krebs cycle would also be valuable in this respect (Table II), since they can be estimated by the more sensitive ninhydrin method (18). Thus, based on citric and malonic acids, spore composition varies with length of storage of the spores. Of great interest is the finding that malonic acid increases when spores are stored two months which would seem to indicate that this acid does not take an active part in resting uredospore metabolism, because acids of proven metabolic participation decrease, e.g. citrate, when these spores are stored.

When these same spores were incubated 2 hours with radiobicarbonate

TABLE II  
FREE AMINO ACIDS OF BEAN RUST UREDOSPORES. DATA ARE EXPRESSED AS  $\mu$ G. ACID PER G. FRESH WEIGHT SPORE AND ARE AVERAGES OF TWO DETERMINATIONS

| Acid                   | $\mu$ G. |
|------------------------|----------|
| Aspartic               | 29.3     |
| Glutamic               | 467.0    |
| Serine                 | 40.6     |
| Glycine                | 124.0    |
| Asparagine             | 87.5     |
| Threonine              | 50.6     |
| Alanine                | 551.0    |
| Glutamine              | 218.5    |
| Lysine                 | 16.5     |
| Proline                | 46.8     |
| Valine                 | 74.9     |
| Leucines               | 65.4     |
| Phenylalanine          | 64.9     |
| $\gamma$ -Aminobutyric | 489.5    |

the various intermediates became labeled as shown in Table III. Most of the radioactivity was found in alanine, glutamic, succinic, malic and fumaric acids. In general, label was widely distributed among components of the spore except that significant label was not found in the sugar or protein amino acid fractions. At the shortest interval of time, 0.15 minute, only malic, aspartic and malonic acids were labeled. Changes in the distribution of label in these intermediates during the time periods studied are depicted graphically in Figure 1. Malic, malonic and aspartic acids must have been derived from an early product of dark  $\text{CO}_2$  fixation which was common to all three, since slopes of  $\text{C}^{14}$  distribution with time were negative (19). Furthermore, this pattern of labeling during short time periods of dark carbon dioxide fixation resembles that found by Saltman *et al.* (20) for higher plants with the additional complication that malonic acid is also being produced. No activity was found in the nonionic fraction consisting for the most part of sugars, nor was significant activity found among the protein amino acids.

Results of the trapping experiment demonstrated that oxalacetic acid became labeled during the course of dark carbon dioxide fixation in uredospores. This was shown both by recrystallization of the oxalacetic acid hydrazone to constant specific activity and the detection of radioactivity in the hydrazone spot after chromatography on filter paper. Identification of the 2,4-dinitrophenylhydrazone of oxalacetic acid was confirmed by infrared spectrophotometry.

The distribution of  $\text{C}^{14}$  among components of the spores after their

TABLE III  
DISTRIBUTION OF  $\text{C}^{14}$  IN ACIDS OF THE UREDOSPORES OF BEAN RUST FUNGUS AT  
DIFFERENT PERIODS AFTER INCUBATION WITH  $\text{NaHC}^{14}\text{O}_3$

| Acid                   | Percentage of radioactivity in each acid after incubation (minutes) |      |      |      |      |
|------------------------|---------------------------------------------------------------------|------|------|------|------|
|                        | 0.15                                                                | 1    | 5    | 10   | 120  |
| Succinic               | 0                                                                   | 4.2  | 5.1  | 17.0 | 35.5 |
| Malic                  | 43.4                                                                | 24.0 | 17.3 | 8.9  | 10.8 |
| Fumaric                | 0                                                                   | 10.6 | 13.1 | 10.4 | 5.8  |
| $\alpha$ -Ketoglutaric | 0                                                                   | 7.2  | 3.8  | 5.5  | 4.8  |
| Citric                 | 0                                                                   | 1.4  | 2.0  | >0   | 0.9  |
| Malonic                | 21.7                                                                | 8.7  | 5.8  | 1.1  | 0.7  |
| Pyrrolidone carboxylic | 0                                                                   | 0    | 3.2  | 4.4  | 4.2  |
| Aspartic               | 34.7                                                                | 36.7 | 32.2 | 22.2 | 1.0  |
| Glutamic               | 0                                                                   | 0    | 3.8  | 11.0 | 8.4  |
| Alanine                | 0                                                                   | 0    | 1.1  | 13.6 | 21.6 |
| Serine                 | 0                                                                   | 0    | 0    | 0    | 0.7  |
| Glycine                | 0                                                                   | 0    | 1.4  | 0    | 1.4  |
| Asparagine             | 0                                                                   | 0    | 0    | 0    | 0.7  |
| Glutamine              | 0                                                                   | 0    | 0    | 0    | 3.4  |
| $\gamma$ -Aminobutyric | 0                                                                   | 0    | 0    | 0    | 0    |
| Tryptophan             | 0                                                                   | 1.5  | 1.0  | 0    | 0    |
| Unknown                | 0                                                                   | 7.3  | 6.8  | 6.0  | 0    |

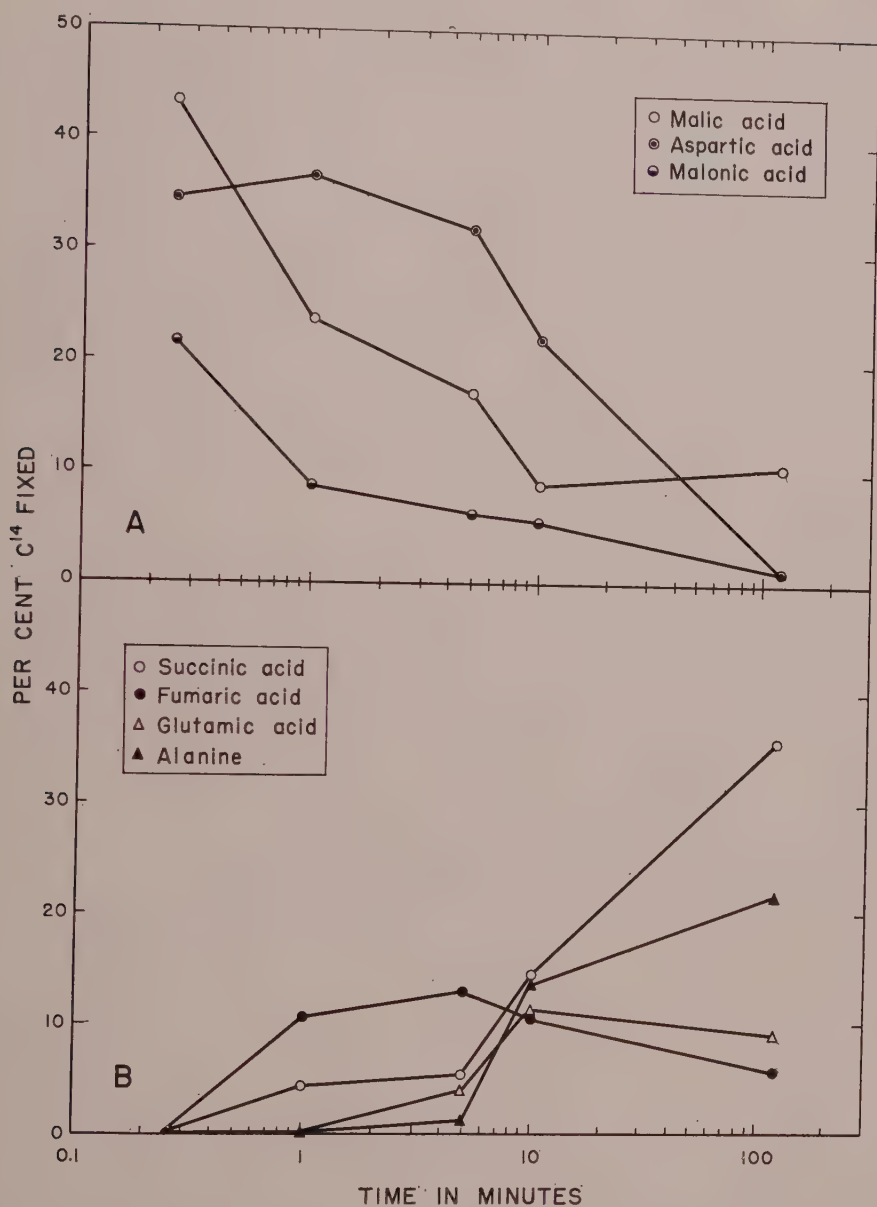


FIGURE 1. Changes in distribution of  $C^{14}$  with time. (A) Three early products of  $CO_2$  fixation in bean rust uredospores. (B) Important acids of bean rust uredospores. None of these acids are early products of  $CO_2$  fixation.



incubation with succinic-1- and fumaric-1- $C^{14}$  acids for 4 hours is depicted in Table IV. Label was distributed among Krebs cycle acids and amino acids associated with the cycle, but not in the sugar or protein fractions. Of special interest is the detection of label from succinic and fumaric acids in malonic acid, so that these acids must be associated with the pathway to malonic acid, a finding of some importance, since they both can yield oxalacetic acid a common member of the Krebs cycle.

Of the total radioactivity found in malate isolated from bean rust uredospores incubated with radiobicarbonate, 38 per cent was in carbon atom 1 while 62 per cent was in carbon atom 4. No radioactivity was found in carbon atoms 2 and 3. This distribution is very similar to that described by Bradbeer, Ranson and Stiller (2) for crassulaceous leaves, adding weight to the contention that oxalacetate is the first product of fixation and that this fixation is via phosphoenolpyruvate following the mechanism postulated by these authors.

*Wheat leaf rust uredospores.* A limited quantity of wheat leaf rust uredospores were available for dark carbon dioxide fixation studies. The data depicting this fixation are presented in Table V. After 2 hours incubation with bicarbonate most of the label was present in the glutamic acid family of amino acids as well as succinic and malic acids with lesser amounts in a broad spectrum of intermediates. Thus after 2 hours the labeling in intermediates of both species of rust spores was qualitatively similar, though the distribution between succinic and malic acids was more

TABLE IV

DISTRIBUTION OF  $C^{14}$  IN ACIDS OF UREDOSPORES FOUR HOURS AFTER THEIR INCUBATION WITH FUMARIC-1- $C^{14}$  ACID AND SUCCINIC-1- $C^{14}$  ACID (VALUES ARE PERCENTAGES OF METABOLIZED TRACER FOUND IN EACH ACID)

| Acid                   | Percentage of $C^{14}$ in acids from |           |                              |
|------------------------|--------------------------------------|-----------|------------------------------|
|                        | Bean rust with                       |           | Wheat rust<br>with succinate |
|                        | Fumarate                             | Succinate |                              |
| Pyrrolidone carboxylic | 0.1                                  | 1.8       | >0                           |
| Succinic               | 74.4                                 | —         | —                            |
| Malic                  | Trace                                | Trace     | 46.3                         |
| Citric                 | 0.3                                  | 0.9       | 2.3                          |
| Malonic                | 0.1                                  | 0.6       | 7.2                          |
| Pyruvic                | 0.3                                  | 2.7       | 15.8                         |
| Fumaric                | —                                    | 19.0      | 2.2                          |
| $\alpha$ -Ketoglutaric | 0                                    | 7.3       | >0                           |
| Aconitic               | 0                                    | 0         | 7.6                          |
| Aspartic               | 0                                    | 40.0      | 6.3                          |
| Glutamic               | 1.0                                  | 8.2       | 0                            |
| Serine                 | 0                                    | 0.5       | 0                            |
| Glycine                | 1.6                                  | 0.6       | 5.4                          |
| $\alpha$ -Alanine      | 14.2                                 | 13.6      | 5.5                          |
| Lysine                 | 0                                    | 1.9       | 0                            |
| Valine                 | 0                                    | 0         | 1.3                          |
| $\beta$ -Alanine       | 7.0                                  | 0         | 0                            |
| $\gamma$ -Aminobutyric | 0                                    | 0.9       | 0                            |

equitable in the wheat rust spores than in the bean rust spores. No significant label was found in sugars or protein amino acids.

During progressively shorter periods of labeling, relative distribution of  $C^{14}$  was changed so that at the shortest time period, 1 minute, label was found mostly in malic acid.

Table IV lists data obtained after incubating leaf rust spores with succinic-1- $C^{14}$  acid for 4 hours. There was wide distribution of label in intermediates of the Krebs cycle and associated amino acids. As with bean rust spores, succinic acid can obviously serve as a precursor of malonic acid, since the latter became radioactive when the spores were incubated with succinate.

#### DISCUSSION

The finding that label is widely distributed in Krebs cycle intermediates after incubation of uredospores with radiobicarbonate for 1 minute provides clear evidence that a pathway for rapid synthesis of these intermediates exists in resting uredospores. Although the sequence of incorporation has not been determined, it seems probable that the Krebs cycle is functional. Isocitrate was never identified, a situation likely to occur if it were present only in catalytic amounts or if isocitrase (4) were active, a strong likelihood since the spores are rich in fat (21). The presence of label in glutamate, aspartate and alanine indicates that transamination reactions also occur. As far as Krebs cycle and associated reactions are concerned, there seems little difference between uredospores and higher plants for even their high content of malonic acid is not unusual (28, 31). Addi-

TABLE V

DISTRIBUTION OF  $C^{14}$  IN COMPOUNDS FROM WHEAT LEAF RUST UREDOSPORES AFTER THEIR INCUBATION WITH RADIOBICARBONATE. DATA ARE EXPRESSED AS PER CENT OF TOTAL RADIOACTIVITY FOUND IN EACH COMPOUND

| Acid                   | Length of incubation (minutes) |      |      |      |
|------------------------|--------------------------------|------|------|------|
|                        | 1                              | 5    | 10   | 120  |
| Succinic               | 4.0                            | —    | 3.6  | 10.1 |
| Malic                  | 59.9                           | 45.9 | 45.2 | 14.7 |
| Fumaric                | 12.1                           | 18.2 | 19.6 | 1.8  |
| $\alpha$ -Ketoglutaric | 6.2                            | 9.7  | 4.7  | 1.0  |
| Citric                 | 4.5                            | 7.2  | 10.7 | 3.8  |
| Malonic                | 1.3                            | 1.9  | >0   | 1.1  |
| Pyrrolidone carboxylic | 0                              | 1.0  | 1.2  | 3.3  |
| Aspartic               | 11.4                           | 5.9  | 2.6  | 0.1  |
| Glutamic               | 0.3                            | 2.7  | 2.8  | 10.4 |
| Alanine                | 0.2                            | 2.3  | 3.9  | 15.7 |
| Serine                 | 0                              | 0    | 0    | 1.2  |
| Glycine                | 0                              | 0.3  | 0    | 3.0  |
| Asparagine             | 0                              | 0    | 0    | 0.5  |
| Glutamine              | 0                              | 0    | 1.0  | 22.7 |
| Valine                 | 0                              | 0    | 0    | 0.5  |
| Methionine sulfoxide   | 0                              | 0    | 0    | 1.1  |
| Unknown                | 0                              | 3.7  | 1.3  | 4.5  |

tional evidence suggesting that the Krebs cycle may operate in these spores is the finding that two acids of the cycle, succinate and fumarate, are precursors for all Krebs cycle acids as well as associated amino acids and malonate. Malonate is thus definitely implicated to be associated with the Krebs cycle.

The early products of dark carbon dioxide fixation were determined to be malonic, malic and aspartic acids. Saltman *et al.* (19) found aspartate and malate to be early products in succulent leaves, and their interpretation was that oxalacetate was the transient first product of  $\text{CO}_2$  fixation onto phosphoenolpyruvate. Oxalacetate also seems to be the first product of  $\text{CO}_2$  fixation in uredospores. The presence of label in oxalacetate as demonstrated using the trapping experiment and the asymmetric distribution of label in malate help to confirm that fixation is completed with oxalacetate formation.

Malonate has been demonstrated to arise from the breakdown of pyrimidines in bacteria (7, 10, 14), but of great interest is a pathway described by Vennesland and Evans (27) who present evidence for the oxidative decarboxylation of oxalacetate to malonate in pig heart brei. This latter is a most likely pathway for malonate production in uredospores in view of the early appearance of label in malonate.

Another source of malonate may prove to be malonyl-coenzyme A, now shown to arise by fixation of carbon dioxide onto acetyl-coenzyme A (29). Malonyl-coenzyme A may be hydrolyzed during passage of the effluent through the strong ion-exchange resins used to produce malonate. Alternately, uredospore metabolism may be partially blocked at the point of fat synthesis and coenzyme A regenerated with malonate production. This point must be investigated further since oxalacetate or malonyl-coenzyme A, both early products of carbon dioxide fixation, could serve as sources of malonate in the manner found here.

Nothing can be said at present for the role of malonate in uredospore metabolism except to suggest that it is not rapidly utilized since it accumulates in spores stored for a period of time while other acids of demonstrated metabolic importance decrease. In bush bean there is also a large accumulation of malonate, yet this carbon does enter the Krebs cycle (31), though the quantitative significance of this has not been determined. It may be that malonate can contribute carbon to the Krebs cycle in uredospores, but that carbon enters the malonate pool faster than in leaves.

Recently, Bradbeer, Ranson and Stiller (2) have degraded malate obtained from crassulaceous leaves after dark  $\text{CO}_2$  fixation was allowed to proceed. An hypothesis involving the carboxylation of phosphoenolpyruvate was put forward by them as the mechanism by which carbon dioxide is fixed in these leaves. Presence of a phosphoenolpyruvate car-

boxylase has not yet been demonstrated in uredospores possibly because of difficulty in disrupting them in any quantity without ball milling. However, degradation of malate isolated from bean rust uredospores has given a distribution of label very similar to that of Bradbeer *et al.* (2), so that carboxylation could be taking place in uredospores via a mechanism like that postulated by them and not via the malate enzyme.

The quantitative significance of the Krebs cycle in endogenous spore metabolism is unknown. After 2 hours, a reasonably long period of incubation with  $\text{NaHC}^{14}\text{O}_3$ , most of the label was distributed in succinate and malate while much less was in citrate. Both spore types were similar in this respect. It would seem that each intermediate acid became labeled by a reversal of the cycle leading to more label in malate rather than citrate. Perhaps label was mostly induced merely by mass action rather than by metabolic movement. This seems to be the case, but, since the amount of carbon dioxide available for fixation may have been very large relative to normal amounts of endogenous acceptor, this backward movement of the label may not truly mirror the importance of the Krebs cycle to endogenous metabolism. For the moment the question is undecided.

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# THE EFFECT OF 2,4-DITHIOBIURET ON SOME OF THE BIOCHEMICAL COMPONENTS OF COTTON AND COLEUS PLANTS

PHILIP H. PLAISTED

## SUMMARY

Dithiobiuret applied to the soil around cotton or coleus plants promoted leaf abscission with no apparent morphological change in the leaves. Leaves from the treated plants contained more protein than the control, 92.5 mg. compared to 72.5 mg./leaf. The protein content of the treated plants remained higher than the control for 72 hours after treatment or until time of abscission. The increase in protein/leaf was believed to be caused by a new type of protein-like material for the ratio of amino acids in the hydrolyzate from the treated leaves was not the same as from the untreated control. The amount of each free amino acid in the treated plants increased from twice to more than 10 times the amount found in the untreated plants.

Dithiobiuret treatment increased the respiration rate about 50 per cent. The respiratory quotients of the leaves ready to abscise and of those on the control were the same, 0.96. Carbohydrates were apparently the substrate for respiration even at time of abscission.

Inorganic phosphorus uptake was accelerated about 200 per cent shortly after treatment but then declined until less phosphorus was found in the treated than in the untreated leaves.

Studies with  $C^{14}O_2$  showed that dithiobiuret treatment accelerated turnover of carbohydrates. The increase in synthetic rate of free amino acids following dithiobiuret treatment was greater among alanine, aspartic and glutamic acids.

Some metabolic implications of dithiobiuret treatment are discussed.

## INTRODUCTION

Dufrenoy and Pratt (9) used 2,4-dithiobiuret to induce root and shoot development in grape cuttings and to lengthen the life of cut daisies. Dufrenoy *et al.* (8) also found that this same compound would promote leafy shoot development on *Ficus gnaphtholocarpa*. The abscission promoting activity of dithiobiuret was used by Goodhue and Stansbury (13) as an activator for polysulfide defoliant formulations.

The present study was undertaken to acquire information on the effect of dithiobiuret on some of the metabolic constituents in plants with special reference to abscission. This compound accelerated leaf abscission in cotton and coleus plants. The leaves that abscised were in most cases green and turgid and showed no morphological damage.

## EXPERIMENTAL METHODS AND RESULTS

The test plants used were 40- to 60-day-old seedlings of cotton, *Gossypium hirsutum* L. variety Coker 100 wilt, and young plants of

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*Coleus Blumei* Benth. Although the coleus and cotton differed in age at time of treatment, both had 8 to 12 leaves per plant. Coleus was used in the winter months when cotton was difficult to grow.

Technical grade 2,4-dithiobiuret was used to induce abscission of all test plants. Abscission was promoted in the plants either by placing one or more grams of the technical powder on the soil surface of the growing container or in one experiment the plants were sprayed with an aqueous solution of the sodium salt. Soil applications made possible biochemical analysis of leaf and stem tissue without danger of contamination from spray residue.

The various methods used and the results obtained from each analysis are discussed under separate headings.

#### OXYGEN CONSUMPTION AND RESPIRATORY QUOTIENT

The oxygen consumption and respiratory quotient were determined in a Warburg micro respirometer on discs cut from treated and untreated cotton leaves by a modification of the method Klinker (16) used for tomato leaves. Leaves corresponding in shape, size and position on both the treated and untreated plants were harvested. Discs were punched from the leaf blades with a No. 4 cork-borer and then floated on cool tap water for an hour before starting the experiment. The leaf discs were placed in the Warburg flask which contained about 20 mg. of moist powdered cellulose. The center well held 0.2 ml. of 10 per cent KOH. Each flask received the same volume of material and the same number of leaf discs. The flasks were sealed, attached to the manometers and tightly wrapped with aluminum foil to exclude all light. After equilibration in the water bath for 30 minutes, the manometers were closed and the experiment was continued for four hours. The CO<sub>2</sub> measurements were made on a series of flasks that contained no KOH in the center well. The amount of CO<sub>2</sub> evolved was determined by the conventional method of difference between the O<sub>2</sub> consumed and the CO<sub>2</sub> evolved (34, pp. 17-20).

The treated plants utilized more O<sub>2</sub> than the untreated controls. At the end of 24 hours leaves from treated plants utilized 804  $\mu$ l. of O<sub>2</sub>/hour/leaf, while the control leaves utilized only 701  $\mu$ l./hour/leaf. Twenty-four hours later, or 48 hours after treatment, the O<sub>2</sub> consumption of the treated leaves had increased to 1173  $\mu$ l./hour/leaf while untreated leaves consumed only 762  $\mu$ l./hour/leaf. At the end of 168 hours after applying dithiobiuret to the soil surface, the leaves abscised when lightly touched. The O<sub>2</sub> consumption of these abscised leaves was 1205  $\mu$ l./hour/leaf. Carbon dioxide evolution was 1150  $\mu$ l./hour/leaf, giving a respiratory quotient (R.Q.) of 0.96. A similar R.Q. of 0.97 was obtained from leaves of the control plant which consumed 820  $\mu$ l. of O<sub>2</sub>/hour/leaf and respired 796  $\mu$ l. of CO<sub>2</sub>. Although the R.Q. was measured only once, this measurement was

on leaves ready to absciss and should be representative of the treated leaves. Both the treated and untreated leaves had an R.Q. of approximately one which would indicate that carbohydrates were the substrate for respiration irrespective of treatment.

#### ENZYMATIC STUDIES

The effect of 2,4-dithiobiuret on the phosphorylation of glucose by adenosine triphosphate (ATP) using the enzyme hexokinase as a catalyst was studied. The enzyme was obtained from Nutritional Biochemical Corporation. The ATP was purchased from Mann Research Laboratories.

The hexokinase reaction was carried out as described by Colowick and Kalckar (6). A series of Warburg flasks was prepared; one set had the complete enzyme system; a second had the complete enzyme system made up in a one-half saturated solution of 2,4-dithiobiuret; the third set contained the complete system including the chemical but no enzyme. The 0.04M ATP in 0.04M NaHCO<sub>3</sub> was tipped in from the side arms after the flask had been gassed with a mixture of 95 per cent nitrogen and 5 per cent CO<sub>2</sub> and was in equilibrium with the water bath. The course of gas evolution was followed for 17 minutes.

The toxicant inhibited the enzymatic reaction about 60 per cent. In systems containing no dithiobiuret 111  $\mu$ l. of CO<sub>2</sub> were produced during the 17-minute period. Systems with one-half saturated solutions of the chemical produced 114  $\mu$ l. of CO<sub>2</sub> whereas a similar system containing no enzyme produced only 69  $\mu$ l. The CO<sub>2</sub> given off in the flask containing the complete system including the chemical but no enzyme could arise from a MgCl<sub>2</sub>-dithiobiuret reaction. The Mg ion, required by the enzymatic system being studied, was supplied as MgCl<sub>2</sub>. Dithiobiuret may chelate (26) part of the Mg ions in the solution which in turn would liberate some CO<sub>2</sub>. If all of the Mg ions were chelated and not available for the reaction, the flasks containing a complete system plus the chemical would give results directly comparable to the complete system minus the enzyme plus the compound. This was not true, therefore some of the Mg ions, either chelated or unchelated, were available for the reaction.

#### ANALYTICAL RESULTS

The naturally occurring chemical components that were studied during the abscission process are discussed below. With each material or group of materials studied is a brief description of the analytical techniques used and the results obtained.

*Extraction of tissue.* The harvested tissue was lyophilized, then ground to pass a 40-mesh screen in a Wiley mill and extracted with hot alcohol. When fresh tissue was used, it was homogenized in 80 per cent ethanol with a VirTis "45" homogenizer for five minutes, centrifuged, and the super-



natant decanted. Four more half-hour extractions were carried out with warm (50° C.) 80 per cent ethanol solution. In the last or sixth extraction, warm water was used, and all extracts were combined. Dry tissue was extracted in a similar manner. The residue was lyophilized, weighed and stored over  $\text{CaCl}_2$ .

*Nitrogen.* Protein nitrogen was determined on the alcohol insoluble residue by the semi-micro Kjeldahl technique (38). The amount of protein per leaf was calculated by multiplying the amount of protein N per leaf by 6.25.

The amino acid composition of the leaf protein was found on an aliquot of the dried alcohol-extracted residue. About 120 mg. of the dried material were sealed in a test tube with 15 ml. of 6*N* HCl and hydrolyzed for 20 hours under 15 pounds steam pressure. The hydrolyzate was filtered, evaporated to dryness four times on a steam bath and then passed through a column of Dowex 50 which was in the hydrogen form. The amino acids were eluted from the column with 4*N*  $\text{NH}_4\text{OH}$ .

The free amino acids were removed from the 80 per cent ethanol solution by passing through a Dowex 50 column as described by Plaisted (23). The eluted free amino acids and amino acids from the protein hydrolyzate were dried *in vacuo* and made up to a known volume in a solution of 10 per cent isopropanol in water. Aliquots from these samples were spotted on large sheets of Whatman No. 3 filter paper and chromatographed. The different amino acids and amides were identified by using different chromatographing solvents in conjunction with special spot tests (3, pp. 91-100). Quantitative amino acid chromatography was carried out as described by Porter *et al.* (27).

Dithiobiuret caused an increase in protein nitrogen in the leaves of the cotton plants. This increase occurred within the first day, for 24 hours after treatment leaves from treated plants had 92.5 mg. of protein/leaf while corresponding untreated leaves had only 72.5 mg./leaf. Forty-eight hours after treatment there were 93.5 mg. of protein/leaf compared to the controls which had 81.9 mg./leaf. Twenty-four hours later, or 72 hours after treatment, the treated leaves had 79.4 mg. of protein while corresponding control leaves had only 60.4 mg. Preliminary information (22) indicated protein breakdown but the more refined analytical techniques used in these experiments show that this did not occur.

The increase in protein content/leaf could arise in two ways, by an increase in the amount of existing protein or by synthesis of a new type of protein. A greater amount of each amino acid was found in the protein hydrolyzate from the treated tissue than from the control plants (Table I). The only exception to this was tyrosine. A better comparison of the amino acid composition of the protein from the treated and control plants can be obtained by comparison of the molar ratio of the different amino

TABLE I  
AMINO ACID COMPOSITION OF PROTEINS FROM COTTON LEAVES TREATED WITH  
2,4-DITHIOBIURET AND THE UNTREATED CONTROL

| Amino acids   | Amino acid composition in   |                               | Molar ratio based on alanine |                  |
|---------------|-----------------------------|-------------------------------|------------------------------|------------------|
|               | Treated leaves,<br>μg./leaf | Untreated leaves,<br>μg./leaf | Treated leaves               | Untreated leaves |
| Alanine       | 304                         | 161                           | 1.00                         | 1.00             |
| Arginine      | 271                         | 148                           | 0.89                         | 0.92             |
| Aspartic acid | 647                         | 200                           | 2.13                         | 1.30             |
| Glutamic acid | 609                         | 386                           | 2.00                         | 2.41             |
| Glycine       | 280                         | 89                            | 0.92                         | 0.56             |
| Histidine     | 124                         | 71                            | 0.41                         | 0.45             |
| The leucines  | 996                         | 790                           | 3.27                         | 4.91             |
| Phenylalanine | 280                         | 152                           | 0.92                         | 0.95             |
| Proline       | 95                          | 74                            | 0.31                         | 0.46             |
| Serine        | 205                         | 129                           | 0.67                         | 0.81             |
| Threonine     | 256                         | 170                           | 0.84                         | 1.06             |
| Tyrosine      | 149                         | 179                           | 0.49                         | 1.12             |
| Valine        | 346                         | 235                           | 1.14                         | 1.56             |

acids using alanine, which can be measured with a high precision (27) as a standard.

The accumulation rate of each free amino acid found in the treated tissue was not the same. Some amino acids such as  $\gamma$ -aminobutyric acid, glutamine, the leucines, threonine and valine built up to an amount in excess of the control within 24 hours of treatment. Others such as alanine, glutamic acid and serine did not accumulate in amounts in excess of the controls until 120 hours after treatment (Table II). Spots for asparagine and glutamine were visible on the chromatograms of the control plants but the amounts present were not sufficient for quantitative measurement. Dithiobiuret increased the amount of asparagine and glutamine manyfold. Other amino acids such as arginine and lysine were present on both chromatograms of the treated and untreated tissue, but were not present in sufficient amounts on either chromatogram to obtain quantitative measurements. Treating the plants with dithiobiuret did not increase the arginine or lysine content of the tissue as it did the other amino acids.

*Organic acids.* The nonvolatile organic acids in the 80 per cent ethanol extract were determined after the amino acids had been quantitatively removed with the Dowex 50 column. The quantity of each organic acid was determined by the gradient elution method of Palmer (20) as modified by Staples (31). The identity of the different acids was established by paper chromatography. The contents of all of the test tubes containing the same acid were combined, the Na ion removed on a Dowex 50 column and the filtrate taken to dryness. The dried acids were redissolved in a

TABLE II  
RATIO OF FREE AMINO ACIDS IN YOUNG COTTON PLANTS TREATED WITH 2,4-DITHIOBIURET TO THE UNTREATED CONTROLS

| Amino acids                 | Ratio* of acids after |          |          |
|-----------------------------|-----------------------|----------|----------|
|                             | 24 Hrs.               | 120 Hrs. | 192 Hrs. |
| Alanine                     | 67                    | 175      | 124      |
| $\gamma$ -Aminobutyric acid | 368                   | 114      | 116      |
| Arginine                    | T**                   | T        | T        |
| Asparagine                  | T                     | P†       | P        |
| Aspartic acid               | 246                   | —        | 278      |
| Glutamic acid               | 61                    | 112      | 291      |
| Glutamine                   | 948                   | P        | P        |
| Glycine                     | 97                    | 100      | 219      |
| The leucines                | 445                   | 515      | 993      |
| Lysine                      | T                     | T        | T        |
| Serine                      | 78                    | 145      | 246      |
| Threonine                   | 122                   | 179      | 348      |
| Valine                      | 666                   | 890      | 895      |

\* Ratio calculated:  $\mu\text{g./g. dry tissue} \frac{\text{treated}}{\text{control}} 100$ .

\*\* T = present in small amounts in both treated and untreated tissue.

† P = present in large quantities in treated tissue but in trace quantities in untreated tissue.

small volume of water, spotted on Whatman No. 1 filter paper and run in solvent F of Stark, Goodban and Owens (32). Authentic samples were either spotted on the paper or co-chromatographed with them. Enough malic acid was obtained from the cotton leaves during the course of the experiments to crystallize by the same technique that Weinstein (36) used for quinic acid.

The cotton plants used contained six nonvolatile organic acids. Malic, citric and succinic acids were identified and the amount of each determined at the end of 24 and 48 hours after treatment, as seen in Table III. Three unidentified organic acids were eluted from Dowex 1 column before succinic acid. Succinic and malic acids were not always separated clearly enough to determine the quantity of each but it was estimated that they were present in the ratio of 10 malic to 1 succinic acid. Both the 24- and 48-hour data showed that there was a greater amount of each organic acid in the treated than in the control plants. Malic acid was the only organic acid that was present in sufficient amounts to isolate and crystallize. The infrared absorption spectrum of the malic acid isolated from the cotton leaf tissue was the same as that obtained from an authentic sample.

*Carbohydrates.* The kind and amount of each free sugar in the leaf tissue was determined on the 80 per cent ethanol extract that had passed through the Dowex 50 and Dowex 1 resin columns. This alcoholic solution was con-

TABLE III

AMOUNT AND RADIOACTIVITY OF ORGANIC ACIDS IN COTTON PLANTS TREATED WITH DITHIOBIURET AND THE UNTREATED CONTROL

| Organic acids         | Plants treated with dithiobiuret, then exposed to $C^{14}O_2$ 22 hours later |         |             |         | Plants exposed to $C^{14}O_2$ , then treated with dithiobiuret. Harvested 48 hours later |         |             |         |
|-----------------------|------------------------------------------------------------------------------|---------|-------------|---------|------------------------------------------------------------------------------------------|---------|-------------|---------|
|                       | $M$ eq./leaf<br>$10^{-4}$                                                    |         | C.p.m./leaf |         | $M$ eq./leaf<br>$10^{-4}$                                                                |         | C.p.m./leaf |         |
|                       | Treated                                                                      | Control | Treated     | Control | Treated                                                                                  | Control | Treated     | Control |
| Unknowns<br>(3 acids) | —                                                                            | —       | 2,393       | 2,250   | —                                                                                        | —       | 2,569       | 1,079   |
| Succinic              | 1,021                                                                        | 778     | 2,757       | 1,529   | 1,696                                                                                    | 225     | 590         | 444     |
| Malic                 |                                                                              |         | 16,314      | 10,386  |                                                                                          |         | 16,714      | 6,222   |
| Citric                | 42                                                                           | 30      | 1,150       | 1,464   | 131                                                                                      | 55      | 1,651       | 655     |

\* C.p.m. = counts per minute.

centrated to a small known volume, usually 5 ml. or less, and an aliquot of this extract, along with authentic samples, was chromatographed on 20×20 cm. sheets of Schleicher and Shuell No. 598 filter paper. The chromatograms were developed in *n*-butanol—acetic acid—water (4:1:5 v/v/v). The sugar spots were located on the dried chromatogram by lightly spraying with 0.2 per cent  $\alpha$ -naphthoresorcinol (1,3-naphthalenediol) in absolute ethanol and phosphoric acid (10:1 v/v) (5). Quantitative determinations of the sugars on the chromatogram were carried out by a modification of the Pavlinova (21) method. Guide strips on each side and in the center of the developed chromatogram were cut out and the sugars located by spraying. These guide strips and the remaining chromatogram were reassembled and the other sugars located on the undeveloped chromatogram by comparison with the locations on the guide strips. These areas were cut out and placed in a funnel on top of glass wool plugs. The pieces of paper were wet with five 3-ml. aliquots of hot (90° to 100° C.) water. The liquid was collected in test tubes and then evaporated by passing a stream of air into the tubes while they were immersed in a warm (50° C.) water bath. The dried sugars in each tube were reacted with anthrone (39) and the quantity of sugar established by comparing the colors produced by the anthrone-sugar reaction with previously determined standard curves.

The amount of starch in the tissue was determined on an aliquot of the alcohol extracted residue by the technique of McCready *et al.* (17).

The free sugars found in cotton were glucose, fructose and sucrose. Others may be present but were not in high enough quantity to be readily identified. Glucose and sucrose were the predominating sugars as seen in



Table IV. The small amounts of free sugars found in the leaf tissue 48 hours after treatment may be the result of poor light conditions. These plants were enclosed in a bell jar and illuminated with two 200-watt incandescent bulbs. The bulbs were turned on approximately 14 hours out of 24. Only slight differences were found in the amount of sugars between leaves from treated and untreated plants. Starch, the reserve carbohydrate, showed the greatest change upon treatment. The treated plants contained less starch per leaf than the untreated as seen in Table IV. The depletion of starch in the treated leaves was probably associated with the high rate of respiration found in these plants. Plants maintained for 48 hours under artificial light were low in starch content (Table IV) and no difference was detected between the treated and untreated plants.

*Phosphorus.* The test plants, *Coleus Blumei*, were grown in a liquid medium in which the commercial fertilizer, Hyponex, was used as a source of nutrients. At the time of treatment, the plants were removed from the nutrient solution, the roots rinsed in distilled water and then placed in a saturated solution of dithiobiuret. The control plants were placed in distilled water. This technique eliminated most of the phosphorus in the external medium. The plants were harvested periodically after treatment, lyophilized, ground to pass a 40-mesh screen and phosphorus determinations made on an aliquot of this tissue. Total and inorganic phosphorus

TABLE IV  
AMOUNT AND RADIOACTIVITY OF SOME CARBOHYDRATES FROM COTTON PLANTS TREATED WITH DITHIOBIURET AND THE UNTREATED CONTROL

| Carbohydrate | Plants treated with dithiobiuret, then exposed to $C^{14}O_2$<br>22 hours later             |         |              |         |
|--------------|---------------------------------------------------------------------------------------------|---------|--------------|---------|
|              | Mg./leaf                                                                                    |         | C.p.m.*/leaf |         |
|              | Treated                                                                                     | Control | Treated      | Control |
| Glucose      | 22                                                                                          | 17      | 25,568       | 13,493  |
| Fructose     | 3                                                                                           | 3       | 11,229       | 6,343   |
| Sucrose      | 10                                                                                          | 11      | 67,329       | 40,500  |
| Starch       | 58                                                                                          | 125     | 29,590       | 20,859  |
| Carbohydrate | Plants exposed to $C^{14}O_2$ , then treated with dithiobiuret.<br>Harvested 48 hours later |         |              |         |
|              | Mg./leaf                                                                                    |         | C.p.m.*/leaf |         |
|              | Treated                                                                                     | Control | Treated      | Control |
| Glucose      | 0.32                                                                                        | 1.68    | 5,348        | 14,116  |
| Fructose     | Trace                                                                                       | Trace   | 12,655       | 63,560  |
| Sucrose      | 0.72                                                                                        | 0.87    | 28,821       | 56,248  |
| Starch       | 2.5                                                                                         | 2.3     | 30,164       | 36,946  |

\* C.p.m. = counts per minute.

were determined on leaf and stem tissue by the technique of Pons, Stansbury and Hoffpauir (25).

Dithiobiuret treatment slightly decreased the organic phosphorus in the leaves (Fig. 1 A). The organic phosphorus of a cm. of treated stem tissue increased from 80 per cent of the control at the end of 24 hours to 125 per cent at the end of 72 hours (Fig. 1 B).

The inorganic phosphorus in both the leaves and stem showed greater response to dithiobiuret treatment than the organic phosphorus (Fig. 1 A, B). The inorganic phosphorus content of the leaves dropped from 200 per cent of the control at the end of 24 hours to 77 per cent of the control 48 hours later or just before abscission. This trend was not found in the stem. A cm. of treated stem tissue contained more inorganic phosphorus from early in the experiment to just prior to abscission. This increase in phosphorus of the treated stem probably came from the leaves but more inorganic phosphorus was lost from the leaves than was accumulated in the stem. Presumably phosphorus in the inorganic form was either accumulating in the roots or leaching into the external medium.

#### USE OF $C^{14}O_2$

*Materials and methods.* In order to study some of the metabolic constituents of the cotton leaves prior to 2,4-dithiobiuret induced abscission, two different experiments were carried out. Twenty-four hours after the soil around young cotton plants was sprinkled with dithiobiuret, they were exposed to 100  $\mu$ c. of  $C^{14}O_2$  for 2.0 hours. As relatively little incorporation of  $C^{14}$  into the protein amino acids was accomplished, a second experiment was carried out, in which plants were exposed to 100  $\mu$ c. of  $C^{14}O_2$  for 2.75 hours, then treated with dithiobiuret. The leaves were harvested 48 hours after chemical treatment.

The total radioactivity found in the free amino acids, amino acids of the protein hydrolyzate, the organic acids and the carbohydrates was determined by plating a small, known quantity of each material on an aluminum planchette before it was chromatographed and counting the activity with a windowless gas flow tube.

The radioactivity in the individual free amino acids as well as in the amino acids obtained from the protein hydrolyzate was found in the following manner. An autoradiogram was made by incubating a paper chromatogram of the amino acids with a 17×14 inch X-ray film for 30 days. At the end of the incubation period, the film was developed and the dark areas were traced onto the original chromatogram. The activity of these individual spots was determined by placing an end-window counting tube (12) over the predetermined area and recording the counts. Following counting, the chromatogram was treated with ninhydrin, 1 per cent in absolute ethanol, and the amino acids identified by position.

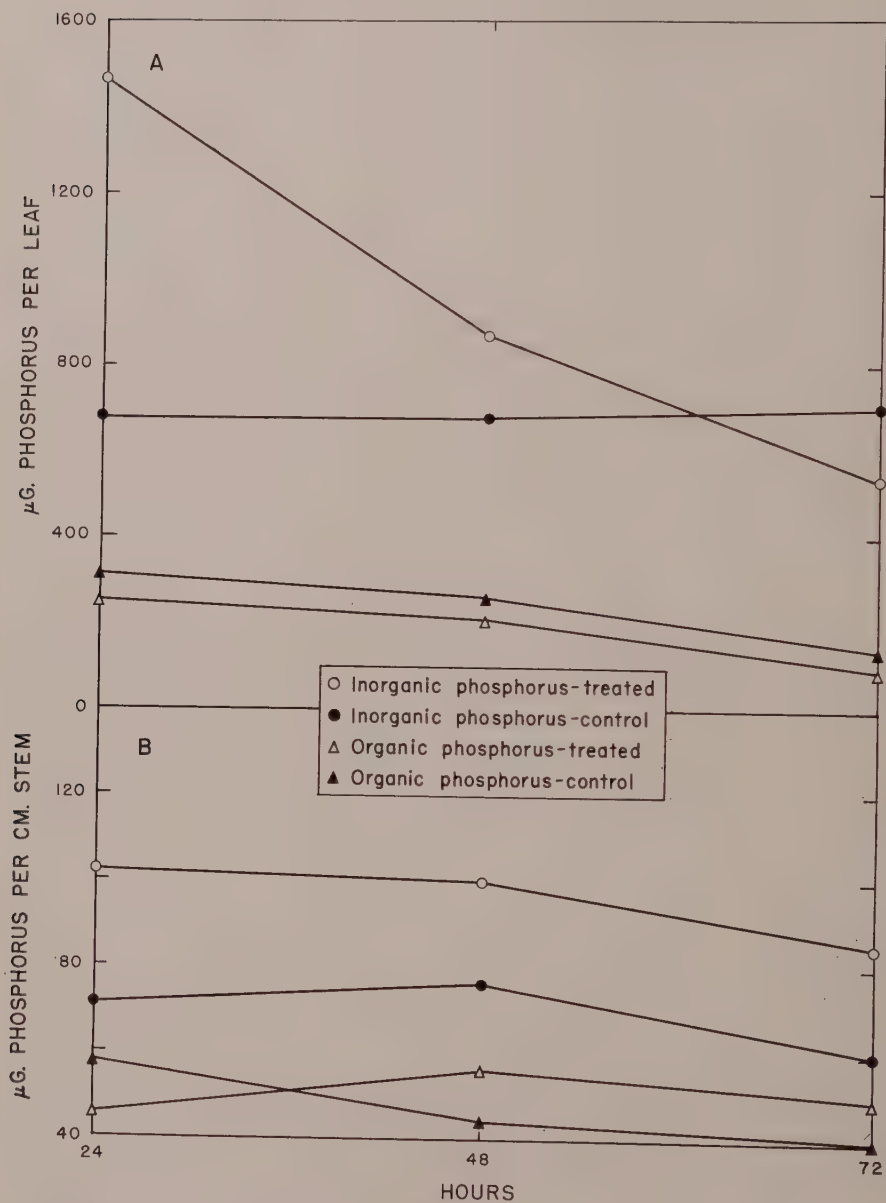


FIGURE 1. A. The effect of dithiobiuret on the organic and inorganic phosphorus content of coleus leaves. B. The effect of dithiobiuret on the organic and inorganic phosphorus content from a centimeter of coleus stem.

The radioactivity of the organic acids was obtained directly from a chromatogram which was developed with solvent F of Stark, Goodban and Owens (32) as previously described. The acids were located by spraying with bromophenol blue indicator, 0.04 per cent in absolute ethanol, and the activity of each acid determined.

The radioactivity of each carbohydrate was determined on a chromatogram. The paper and solvents used to develop the chromatograms were the same as described for carbohydrate analysis. The sugars were located by spraying the paper with  $\alpha$ -naphthoresorcinol and phosphoric acid (5) and the radioactivity determined as previously described.

*Results.* The cotton plants treated with dithiobiuret 22 hours before exposure to  $C^{14}O_2$  showed no morphological evidence of toxicity at the end of the treatment period.

The total activity of the individual amino acids (Tables V, VI and VII), organic acids (Table III) and carbohydrates (Table IV) is not the same as reported in Table VIII. These differences were caused by absorption of the activity by the paper chromatogram. However, the absorption should be equal or nearly equal for each compound. Therefore the results reported in Tables III, IV, V, VI and VII should be valid for comparative purposes.

The incorporation of  $C^{14}$  into the free amino acids was greatest in those

TABLE V

AMOUNT AND RADIOACTIVITY OF THE FREE AMINO ACIDS FOUND IN YOUNG COTTON PLANTS EXPOSED TO  $C^{14}O_2$  22 HOURS AFTER TREATMENT WITH 2,4-DITHIOBIURET

| Amino acids                 | $\mu G./leaf$ |         | Counts/minute/leaf |         |
|-----------------------------|---------------|---------|--------------------|---------|
|                             | Treated       | Control | Treated            | Control |
| Alanine                     | 204           | 93      | 1,564              | 1,179   |
| $\gamma$ -Aminobutyric acid | 428           | 39      | 1,351              | 601     |
| Arginine                    | 31            | —       | 0*                 | 0       |
| Asparagine                  | 207           | 93      | 849                | 532     |
| Aspartic acid               | 357           | 450     | 2,569              | 1,484   |
| Glutamic acid               | 793           | 293     | 2,786              | 3,136   |
| Glutamine                   | 153           | 38      | 439                | 158     |
| Glycine                     | 7             | 3       | 0                  | 0       |
| The leucines                | 171           | 14      | 0                  | 0       |
| Lysine                      | 54            | —       | 0                  | 0       |
| Phenylalanine               | 100           | —       | 0                  | 0       |
| Serine                      | 60            | 24      | 0                  | 0       |
| Threonine                   | 114           | 49      | 0                  | 0       |
| Tyrosine                    | 29            | —       | 0                  | 0       |
| Valine                      | 142           | 14      | 89                 | 0       |
| Tryptophane**               | —             | —       | 286                | 0       |

\* = Radioactivity was too low to measure.

\*\* = Identified from chromatogram Rf only.



TABLE VI

AMOUNT AND RADIOACTIVITY OF THE FREE AMINO ACIDS FOUND IN YOUNG COTTON PLANTS WHICH WERE EXPOSED TO  $C^{14}O_2$ , THEN TREATED WITH 2,4-DITHIOBIURET. PLANTS HARVESTED 24 HOURS LATER

| Amino acids                 | $\mu G./leaf$ |         | Counts/minute/leaf |         |
|-----------------------------|---------------|---------|--------------------|---------|
|                             | Treated       | Control | Treated            | Control |
| Alanine                     | 32            | 42      | 290                | 867     |
| $\gamma$ -Aminobutyric acid | 19            | 37      | T*                 | T       |
| Asparagine                  | T             | 20      | T                  | T       |
| Aspartic acid               | 121           | 70      | 861                | 1,907   |
| Glutamic acid               | 346           | 227     | 1,574              | 3,030   |
| Glutamine                   | 114           | 113     | T                  | T       |
| Glycine                     | 4             | 5       | 131                | 209     |
| The leucines                | 56            | 35      | 0**                | 0       |
| Proline                     | T             | T       | 3,611              | 15,000  |
| Serine                      | 30            | 30      | 169                | 209     |
| Threonine                   | 23            | 22      | 0                  | 0       |
| Valine                      | 23            | 18      | 0                  | 0       |
| Unknowns (5 separate spots) |               | 1,275   |                    | 2,997   |

\* = Trace amounts, visible but not measurable.

\*\* = Radioactivity was too low to measure.

which are considered among the first products of photosynthesis (2, pp. 73-74), alanine, glutamic and aspartic acids as seen in Table V. The radioactivity of the other free amino acids was negligible except that of  $\gamma$ -aminobutyric. The close chemical relationship between glutamic and  $\gamma$ -aminobutyric acid suggests that the high incorporation of  $C^{14}$  into  $\gamma$ -aminobutyric acid was through glutamic acid.

TABLE VII

RADIOACTIVITY OF AMINO ACIDS FROM A PROTEIN HYDROLYZATE OF COTTON LEAVES. THE PLANTS WERE EXPOSED TO  $C^{14}O_2$ , THEN TREATED WITH 2,4-DITHIOBIURET AND HARVESTED 48 HOURS LATER

| Amino acids      | Treated            | Control            |
|------------------|--------------------|--------------------|
|                  | Counts/minute/leaf | Counts/minute/leaf |
| Alanine          | 2,155              | 2,921              |
| $\beta$ -Alanine | T*                 | T                  |
| Aspartic acid    | 457                | 1,132              |
| Glycine          | 1,870              | 878                |
| Glutamic acid    | 955                | 850                |
| The leucines     | T                  | T                  |
| Phenylalanine    | 1,584              | 923                |
| Proline          | T                  | T                  |
| Serine           | 1,480              | 1,304              |
| Valine           | T                  | 0**                |

\* = Trace amounts, visible but not measured.

\*\* = Radioactivity was too low to measure.

TABLE VIII  
TOTAL RADIOACTIVITY OF SOME METABOLITES FOUND IN COTTON LEAVES

| Metabolite      | Plants treated with dithioburet,<br>then exposed to $C^{14}O_2$ 22 hours<br>later |     |             |    | Plants exposed to $C^{14}O_2$ , then<br>treated with dithiobiuret.<br>Harvested 48 hours later |     |             |     |
|-----------------|-----------------------------------------------------------------------------------|-----|-------------|----|------------------------------------------------------------------------------------------------|-----|-------------|-----|
|                 | Treated                                                                           |     | Control     |    | Treated                                                                                        |     | Control     |     |
|                 | C.p.m./leaf                                                                       | %   | C.p.m./leaf | %  | C.p.m./leaf                                                                                    | %   | C.p.m./leaf | %   |
| Free amino acid | 18,428                                                                            | 5   | 14,857      | 7  | 51,829                                                                                         | 11  | 121,520     | 16  |
| Organic acid    | 22,628                                                                            | 6   | 15,614      | 7  | 20,995                                                                                         | 5   | 8,005       | 1   |
| Protein         | 31,000                                                                            | 8   | 38,548      | 18 | 167,724                                                                                        | 36  | 174,552     | 23  |
| Sugars          | 271,371                                                                           | 73  | 121,457     | 57 | 194,657                                                                                        | 42  | 425,240     | 56  |
| Starch          | 29,714                                                                            | 8   | 20,857      | 10 | 30,164                                                                                         | 7   | 36,946      | 5   |
| Total           | 373,141                                                                           | 100 | 211,333     | 99 | 465,369                                                                                        | 101 | 766,265     | 101 |

\* C.p.m. = counts per minute.

The major free carbohydrates, glucose, fructose and sucrose, were present in both the treated and untreated plants in about the same amounts, as seen in Table IV. About twice as much radioactivity was incorporated into the treated as the untreated leaves. The reverse of this was found in the starch. More starch was found in the control than in the treated plants, yet Table IV shows that a slightly greater amount of radioactivity was incorporated into the starch of the treated plants. These data suggest that carbohydrate metabolism was accelerated in the treated plants.

Cotton plants exposed to  $C^{14}O_2$ , then treated with dithiobiuret, contained about one-half as much total activity at the end of 48 hours as the untreated control (Table VIII). A detailed analysis of the radioactivity in these plants shows that the free amino acids (Table V), and sugars (Table IV) of the control contain more  $C^{14}$  than the treated, while the organic acids (Table III) of the treated plants contain more radioactivity than the controls. The amino acids of the protein hydrolyzate (Table VII) and starch (Table IV) contained about the same amount of  $C^{14}$  in both the treated and untreated plants.

The small amounts of carbohydrates and free amino acids found in these plants were probably caused by the low light intensity used. After treating with  $C^{14}O_2$ , then with dithiobiuret, the plants were illuminated with two 200-watt incandescent bulbs for 14 out of each 24 hours. The bulk of the radiation emitted by these bulbs was in the red and far red part of the spectrum which was not conducive to photosynthesis.

The greatest radioactivity in the free amino acids of plants harvested 48 hours after becoming radioactive was in proline and glutamic acid (Table VI). A relationship between glutamic acid and proline has been suggested with one of the intermediates, pyrrolidonecarboxylic acid (33). This reaction could account for the high activity found in proline.

The organic acids in the treated plant contained more  $C^{14}$  and were present in greater amounts than in the control (Table III). Initially both plants contained about the same amounts of each organic acid; therefore, the build-up of  $C^{14}$  in the malic and succinic acids of the treated plants was less when expressed as counts per minute per milliequivalent of acid than the check. Presumably much of the malic and succinic acids was derived from nonradioactive material. Citric acid of both treated and untreated plants contained about the same specific activity.

The lower amount of  $C^{14}$  in the carbohydrates of the treated plants (see Table IV) would indicate a rapid turnover of activity caused by treatment. Although less glucose was found in the treated plants, 0.32 mg./leaf as compared to 1.68, the specific activity was greater.

#### DISCUSSION

Respiration of plants treated with 2,4-dithiobiuret increased about 50 per cent above the control and remained high until the leaves dropped. A similar increase in respiration was found by Rasmussen (28) in the roots of dandelions when either 2,4-dichlorophenoxyacetic acid (2,4-D) or kerosene was applied to the tops. Hall and Lane (14) showed that the defoliants, Shed-a-Leaf<sup>1</sup>, Endothal<sup>2</sup> and ethylene, increased the respiration rate of cotton leaves. Apparently the application of many toxic compounds to plants increases the respiration rate as does dithiobiuret.

The R.Q. of treated leaves ready to absciss and untreated control leaves was essentially the same, 0.96 and 0.97. A R.Q. of approximately one shows that carbohydrates were the substrate for respiration even at time of abscission. The amount of carbohydrates found in a leaf at time of abscission is not believed to play a significant role in the abscission process. Hall and Lane (14) reported that the amount of carbohydrates present in cotton leaves at time of chemically induced abscission was high in some experiments and low in others. Plaisted (24) reported adequate carbohydrates in maple leaves at time of natural abscission. Although the amount of carbohydrates *per se* may not be important, the more rapid availability of their breakdown products by an increase in respiration rate may play an important part in the abscission process.

The application of dithiobiuret to the soil around young cotton plants caused an increase in protein or protein-like material in the leaf. Whether the molecule of dithiobiuret, which contains three atoms of reduced nitrogen, entered directly into protein formation or whether the plant utilized the atoms of reduced nitrogen after the dithiobiuret molecule was broken down was not demonstrated by this series of experiments. Irrespective of how the compound caused a protein increase in the leaf, a relatively large

<sup>1</sup> Active ingredient is magnesium chlorate.

<sup>2</sup> "Endothal" is the generic name for disodium 3,6-en doxohexhydrophthalate.

amount of a new type of protein was formed, a unique situation in plants. This protein or protein-like material was considered to be new, for the ratio of the different amino acids in the tissue hydrolyzate was not the same from treated as from untreated plants (Table I).

The increase in plant protein by chemical application was not a new finding. Freiberg and Clark (11) demonstrated an increase in protein content of soybean stem after 2,4-D application. Sell *et al.* (30) reported that the protein in stem of red kidney beans was increased by treating the plant with 2,4-D. Borthwick *et al.* (4) in 1937 reported that indoleacetic acid increased protein in stems of tomato plants in areas where cell division was most rapid. Application of 2,4-D to beans can cause initiation of a large number of small, meristematic-like cells. These cells would be richer in protein than the corresponding area of the stem on a control plant. This could account for the increased protein after 2,4-D applications. Hydrolysis of this protein may present a different ratio of amino acids than hydrolysis of protein from the same area on the control plant (30). In the 2,4-D treated plants the comparison was between the type of protein in old and new cells while the comparison in dithiobiuret-treated plants was between protein in the same type of cells. For this reason the increase in "new" protein after dithiobiuret treatment is considered unique.

Dithiobiuret treatment caused an initial increase of inorganic phosphorus followed by a sharp decline with only a slight increase of organic phosphorus. This increase in phosphorus found with dithiobiuret treatment was similar to that found by Rebstock *et al.* (29) when cranberry beans were treated with 2,4-D. The pattern of phosphorus distribution varies with the plants, for 2,4-D treated tobacco plants showed less phosphorus in the tops than the untreated controls (37).

The increase of phosphorus in dithiobiuret-treated plants could arise from a more rapid uptake from the soil.

The high respiration rate found in treated leaves could aid in accumulating phosphorus. Epstein (10) pointed out the need for respiratory energy and intermediates of the Krebs cycle for absorption of inorganic phosphorus from the soil. Both the respiratory energy and Krebs cycle intermediates would be readily available in the treated plants.

The effect of dithiobiuret on enzymatic systems was not intensively investigated although partial inhibition of the hexokinase reaction was demonstrated. Owens (19) demonstrated that dithiobiuret inhibits polyphenol oxidases. Dawson and Tarpley (7) report that sulfhydryl compounds can inhibit some copper oxidases. Biuret is a good complexing agent for many metals (18). Possibly dithiobiuret could act in a similar fashion by chelating metallic atoms which would either inhibit or accelerate different enzyme systems (15).

The effect of dithiobiuret was greatest in the systems associated with



synthesis and breakdown of the carbohydrates. When plants were treated with dithiobiuret and 22 hours later exposed to  $C^{14}O_2$ , the majority of the activity was located in the sugar and starch fractions; 81 per cent for the treated, 67 per cent for the controls. Plants that were made radioactive, then treated with dithiobiuret, showed a similar trend. The carbohydrate and starch fractions accounted for 47 per cent activity in the treated and 61 per cent in the controls. In the latter case both plants had about the same degree of radioactivity before application of dithiobiuret. The plant which utilized sugars the fastest would have the least radioactivity in the sugar fraction 48 hours after treatment as was the case with the treated plants.

The incorporation of  $C^{14}$  into the free amino acids indicated that dithiobiuret treatment caused a general increase in the rate of free amino acid synthesis. More radioactivity was incorporated into alanine,  $\gamma$ -aminobutyric acid, asparagine, aspartic acid and glutamine when plants previously treated with dithiobiuret were exposed to  $C^{14}O_2$  than in the same amino acids of the untreated control. Less radioactivity was found in the glutamic acid of the treated plants than in the control. This does not necessarily mean that glutamic acid was being formed at a slower rate in the treated than in the control. Those amino acids which probably were derived from glutamic acid,  $\gamma$ -aminobutyric acid and glutamine, contained a relatively high degree of activity. In fact, when the plants were made radioactive by exposure to  $C^{14}O_2$  and treated with dithiobiuret, glutamic acid contained one-half the activity of the control. This would show that glutamic acid was being metabolized faster in the treated plant than in the control.

The increase in radioactivity of aspartic and glutamic acids in the dithiobiuret-treated plants was similar to that found in 2,4-D treated bean plants by Akers and Fang (1). An increase of 50 to 60 per cent in radioactivity of aspartic and glutamic acids was caused by 2,4-D treatment in the beans. Dithiobiuret caused an increase of about 50 per cent in the radioactivity of aspartic acid and rapid side reactions of glutamic acid which might mean that 2,4-D and dithiobiuret do not act primarily on free amino acid synthesis. The rate of amino acid synthesis may be influenced more by the abundance of precursors than by the toxicants themselves.

The total incorporation of  $C^{14}$  into the amino acids found in the protein hydrolyzate was about the same in both the treated and untreated plants. Even though the total radioactivity of the two different protein hydrolyzates was the same, the distribution of the activity was not the same. Alanine and aspartic acid were less radioactive than the other amino acids. This could indicate that these were readily metabolized to form other amino acids or other compounds. This accelerated metabolism of

alanine and aspartic acid in dithiobiuret treated plants may arise as part of the general increase in metabolic rate or it may indicate that these amino acids are part of a metabolic pattern which was accelerated faster than the rest of the system.

Plants treated with dithiobiuret show accelerated amino acid synthesis and greater accumulation of protein than the control plants. Yet the chemical analogue of dithiobiuret, biuret, causes a decrease in protein synthesis (35). The replacement of oxygen by sulfur in the biuret molecule produces a different biological reaction, yet both compounds, if left in contact with the plant long enough and in high enough amounts, will cause death.

Dithiobiuret apparently causes a speed-up in many of the metabolic reactions, probably those associated with leaf abscission.

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*Note*

THE APPROXIMATE AMINO ACID COMPOSITION OF  
WILD AND HATCHERY TROUT (*SALVELINUS*  
*FONTINALIS*) AND SOME OF THEIR PRINCIPAL  
FOODS (*GAMMARUS* AND *HEXAGENIA*  
*BILINEATA*)<sup>1</sup>

RICHARD J. BLOCK

Little is known about the pattern of amino acids required for optimal nutrition of brook trout. In order to obtain some evidence in this connection, two approaches have been employed: the first is to estimate the pattern of amino acids furnished wild fish consuming insects, the second is to estimate the amino acid composition of both wild and hatchery raised trout. The rationale of the latter approach is based on experience in animal husbandry, namely, that if the amino acid pattern of the diet approximates that of the animal to be fed, improved nutrition results with a minimum of protein consumed. The amino acid composition of the principal insect foods, of a practical artificial diet, and of both wild and hatchery trout is reported here. All the materials used in this investigation were supplied through the kindness of Dr. Arthur M. Phillips, Jr., Fish and Wildlife Service, United States Department of the Interior, Cortland, New York.

EXPERIMENTAL

The samples of scuds (*Gammarus*), may flies (*Hexagenia bilineata*), mixed insects, artificial food (5), or whole trout were ground in acetone and treated with organic solvents to extract the water and lipids (1, p. 14). The dry material was hydrolyzed with 6*N* HCl under reflux and the amino acids in the hydrolyzate were estimated by paper chromatography (1, pp. 71-83).

RESULTS AND DISCUSSION

The analytical values are given in Table I.

The amino acid composition of large and small, wild and hatchery trout appears to be the same within the experimental errors of the method. It should be noted that insect proteins seem to contain greater quantities of arginine and cystine and smaller amounts of methionine, threonine, glycine and alanine than those of the trout. Hydroxyproline is apparently not present in insects presumably due to the absence of collagen.

<sup>1</sup> Aided in part by a grant from the Special Products Division, Standard Brands, Inc.

TABLE I

APPROXIMATE AMINO ACID COMPOSITION OF WILD AND CULTIVATED TROUT AND THEIR NATURAL AND ARTIFICIAL FOODS. AMINO ACIDS IN G. PER 16.0 G. OF N IN THE HYDROLYZATE

| Amino acid      | Natural food |           |               | Artificial food | Trout             |                         |                        | Salmon diet (2) |
|-----------------|--------------|-----------|---------------|-----------------|-------------------|-------------------------|------------------------|-----------------|
|                 | Scuds        | May flies | Mixed insects |                 | Wild, fingerlings | Hatchery Penn. (9.6 g.) | Berlin, N.H. (14.3 g.) |                 |
| Arginine        | 6.7          | 6.9       | 5.1           | 6.7             | 4.7               | 4.5                     | 4.6                    | 6.0             |
| Histidine       | 2.6          | 2.7       | 2.9           | 2.7             | —                 | —                       | —                      | 2.7             |
| Lysine          | 6.4          | 6.2       | 5.4           | 5.5             | 6.7               | 6.6                     | 6.8                    | 5.8             |
| Tyrosine        | 3.4          | 5.7       | 5.6           | 3.3             | 3.1               | 3.0                     | 2.9                    | 5.8             |
| Tryptophan      | 1.1          | 1.3       | 1.2           | 0.5             | —                 | —                       | —                      | 1.5             |
| Phenylalanine   | 5.2          | 4.6       | 4.1           | 3.6             | 3.8               | 3.9                     | 3.6                    | 5.8             |
| Cystine         | 3.5          | 3.6       | 3.2           | 1.4             | 1.9               | 1.7                     | 1.7                    | 0.7             |
| Methionine      | 1.3          | 1.4       | 1.3           | 2.0             | 2.0               | 2.0                     | 2.3                    | 2.9             |
| Serine          | 4.0          | 3.6       | 3.5           | 3.1             | 4.6               | 4.5                     | 4.8                    | 4.4             |
| Threonine       | 4.0          | 4.1       | 3.5           | 3.6             | 5.5               | 5.6                     | 5.6                    | 3.6             |
| Leucine         | 5.6          | 5.9       | 4.9           | } 13.0          | 13.4              | 13.3                    | 13.0                   | 8.7             |
| Isoleucine      | 4.5          | 5.4       | 4.3           |                 |                   |                         |                        | 5.8             |
| Valine          | 4.1          | 5.4       | 4.7           |                 |                   |                         |                        | 5.8             |
| Glutamic acid   | 11.2         | 10.7      | 8.6           | 6.5             | 10.9              | 10.9                    | 10.7                   | 11.6            |
| Aspartic acid   | 8.7          | 7.6       | 7.0           | 4.4             | 8.5               | 9.1                     | 8.6                    | 7.3             |
| Glycine         | 4.2          | 3.8       | 3.4           | 3.8             | 9.4               | 8.2                     | 9.2                    | 7.3             |
| Alanine         | 4.6          | 6.3       | 5.9           | 8.6             | 8.6               | 7.8                     | 8.6                    | 5.1             |
| Proline         | 4.2          | 5.6       | 4.9           | —               | 5.0               | 4.5                     | 4.6                    | 7.3             |
| Hydroxy-proline | 0            | 0         | 0             | —               | 1.4               | 1.4                     | 1.6                    | —               |

Halver has shown that Chinook salmon fingerlings (*Oncorhynchus tshawytscha*) grew well on a synthetic diet in which amino acids composed 70 per cent of its dry weight (2). The approximate pattern of the amino acids in this ration is given in Table I. In order to compare Halver's figures with the analytical results, it was assumed that almost all of the glutamic and aspartic acids in proteins are present as the amide. Halver *et al.* (3, 4) have also shown that, like the rat (1), arginine, histidine, lysine, tryptophan, phenylalanine, threonine, leucine, isoleucine and valine are indispensable amino acids for salmon fingerlings and that they require approximately 5.0 g. of lysine and 2.3 g. of threonine per 16.0 g. N for optimal nutrition (4).

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## Note

### BISPORANGIATE CONES IN LARCH

CLYDE CHANDLER

The strobili of the coniferales are usually monosporangiate, the two kinds occurring on the same plant in the Pinaceae and Taxodiaceae and occasionally in the Cupressaceae and Araucariaceae or on different plants which is the prevailing condition in the Cupressaceae and Araucariaceae. Chamberlain (1) in reviewing the literature on bisporangiate strobili states that this condition is most frequently reported in *Picea excelsa*, *Abies alba*, *Pinus laricio* and *Pinus maritima*. They have also been reported in other species of *Pinus* and *Picea*, and in *Pseudotsuga taxifolia*, *Sequoia sempervirens* and *Juniperus communis*. Goebel (2, p. 471) found hundreds of bisporangiate strobili on one tree of *Pinus maritima*. These teratological cones appeared near the apex of the twig and were considered to be male flowers transformed into female ones. At the point of transition he found not infrequently a stamen with a rudimentary ovuliferous scale in its axil. Kirkwood (3) observed abnormal cones among normal ones on a single tree of *Larix occidentalis*. The lower half of the cone presented the normal appearance of the staminate cone with the total absence of the scale. The upper half presented the typical condition of the macrostrobilus. Between the lower and the upper portion of the cone were a few transitional structures representing abortive bracts and scales.

Androgynous cones have been described for *Larix microcarpa* (5, p. 192). The strobili of *Larix* are typically monosporangiate, the two kinds occurring on the same plant.

In 1958, twenty-six bisporangiate strobili were observed on one tree of *Larix leptolepis* Gord. growing at Yonkers, N. Y. Eleven of these strobili were dissected and the number of ovulate and staminate sporophylls were recorded as shown in Table I. The number of needles at the base of the strobilus ranges from 1 to 25. Megasporangiate sporophylls usually occupy the lower portion of the strobilus and the microsporangiate sporophylls are produced on the terminal portion (Fig. 1 C). This condition is the reverse of the arrangement as figured for *Pinus maritima* and described for *Juniperus communis* (1) and *Larix occidentalis* which have the microsporangia near the base and the megasporangia on the upper portion of the strobili. However, two larch strobili were observed with one and three microsporophylls at the base of the strobilus (Table I, strobili 6 and 7). One strobilus terminated in a sporophyll with aborted sporangia (Table I, strobilus 6). Aborted sporangia usually appeared near the base

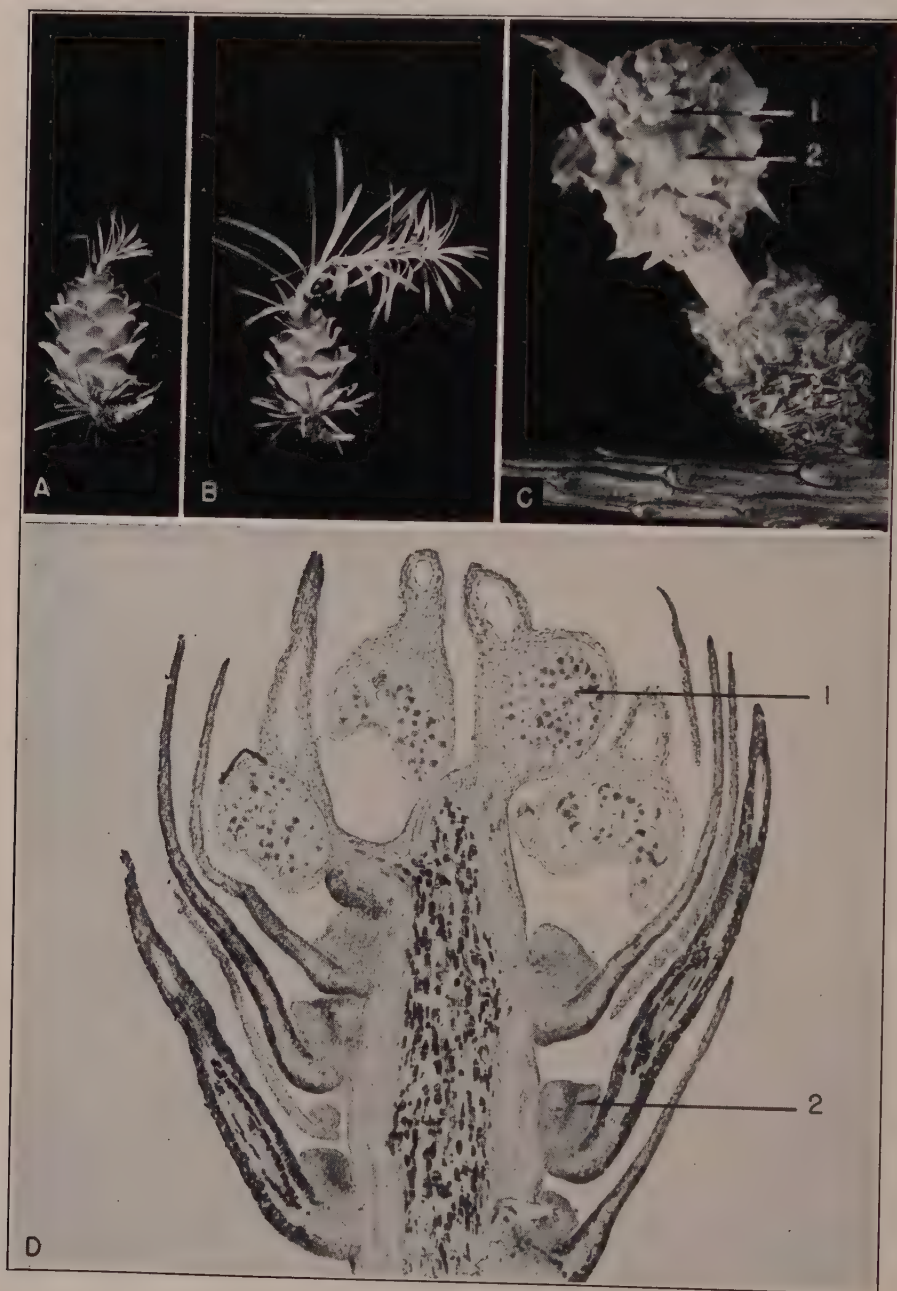


FIGURE 1. (A, B) Proliferated cones of *Larix leptolepis* ( $\times 0.75$ ); (C) bisporangiate strobilus of *Larix leptolepis* ( $\times 3.15$ ), (1) microsporophyll, (2) megasporophyll; (D) longitudinal section through bisporangiate strobilus ( $\times 22.5$ ), microsporangium at apex (1), and megasporangium at base (2).

TABLE I  
BISPORANGIATE STROBILI IN LARCH

| Strobilus examined | No. of needles at base of strobilus | Type of sporophyll* from base to apex of strobilus |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|--------------------|-------------------------------------|----------------------------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1                  | 4                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 2                  | 14                                  | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 3                  | 25                                  | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 4                  | 9                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 5                  | 3                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 6                  | 1                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 7                  | 6                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 8                  | 5                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 9                  | 5                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 10                 | 6                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
| 11                 | 1                                   | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |
|                    | 14                                  | F                                                  | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F |

\* F = megasporophyll.  
M = microsporophyll.  
H = sporophyll with both megasporangium and microsporangium.  
a = sporophyll with aborted sporangia.

TABLE II  
SEEDS PRODUCED IN BISPORANGIATE CONES

| Cones | Total No. of seed | No. of seed with embryo |
|-------|-------------------|-------------------------|
| 1     | 15                | 0                       |
| 2     | 14                | 0                       |
| 3     | 23                | 2                       |
| 4     | 27                | 4                       |
| 5     | 20                | 2                       |
| 6     | 20                | 0                       |
| 7     | 12                | 0                       |
| 8     | 25                | 0                       |
| 9     | 5                 | 0                       |
| 10    | 6                 | 0                       |



of the strobilus or interspersed among megasporophylls. Sporophylls were noted which bore both a mega- and a microsporangium (Table I, strobili 5, 6, 7, 8, 10 and 11).

Five strobili fixed in formalin-propiono-alcohol, embedded in paraffin, sectioned longitudinally at  $15\ \mu$  and stained with safranin and fast green FCF showed very clearly the distribution of the mega- and microsporangia (Fig. 1 D). There was little or no abortion of the mega- and microspores. These bisporangiate strobili are larger than those with microsporangia only but are less than one-half the size of well-developed megasporangiate strobili.

Ten bisporangiate strobili were self-pollinated, tagged and covered with paper bags to determine whether the mega- and microspores were functional in fertilization and the production of well-filled seed. At maturity, when the sporophylls were reflexed, the ten cones were collected, the seed were removed and dissected, and the numbers with and without embryos were recorded as shown in Table II. Most of the seed were empty; only eight well-developed embryos were observed. This is in contrast to normal cones which produce at least 50 per cent viable seeds.

Two proliferated cones, as described for Douglas-fir (4), were observed on the same tree along with the bisporangiate strobili (Fig. 1 A, B). In these cones all sporophylls bore megasporangia only. At maturity one cone had 33 seed, two of which had embryos, and the other cone had 41 seed, three of which had embryos. Only one cone of this type was observed on *Larix laricina* Duroi. Free-hand longitudinal sections of these cones showed the proliferation to be an extension of the main axis of the strobilus.

Published information on bisporangiate cones is very meager. Further morphological studies on the disposition of the sporangia in bisporangiate cones in *Larix* may furnish interesting data for phyletic consideration.

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# THE INFLUENCE OF GIBBERELLIC ACID UPON THE ENDOGENOUS GROWTH SUBSTANCES OF THE ALASKA PEA

I. D. J. PHILLIPS<sup>1</sup>, A. J. VLITOS<sup>2</sup> AND H. CUTLER<sup>3</sup>

## SUMMARY

The amount of endogenous growth substance(s) in pea seedlings (*Pisum sativum* L. var. Alaska) after exposure to red light, darkness, and gibberellic acid (GA) was determined by extracting the plant tissues, separating the materials on paper chromatograms, and assaying their activity by measuring the elongation response of excised first internodes of etiolated oat seedlings (*Avena sativa* L. var. Brighton).

In a series of plants grown continuously in the dark or in red light the relative amounts of growth substance(s) changed with age. The plants in red light had less growth substance(s) than those in the dark in the first few days of growth, about the same amount after 8 to 11 days, and distinctly more after 21 days.

The growth-promoting zones on papers developed with isopropanol—ammonia—water (80:5:15 v/v/v) were found at Rf 0.5 to 0.6 and Rf 0.8 to 0.9. The growth-promoting activity at Rf 0.5 to 0.6 corresponded to an Ehrlich-positive reaction on some of the chromatograms, and also to the position of an authentic 3-indoleacetic acid (IAA) sample concurrently developed on a control strip. However, some of the areas of growth promotion were outside the Ehrlich-positive sectors.

The qualitative patterns of growth-substance activity differed depending upon the age of the seedling. The growth-promoting zone situated at Rf 0.8 to 0.9 appeared markedly only in the seven-day-old seedlings grown in darkness and in eleven-day-old seedlings grown in red light.

Five-day-old Alaska pea seedlings sprayed with 10 p.p.m. solutions of GA and grown under red light showed a 180 per cent increase in the quantity of extractable growth substances after 24 hours. An increase of approximately 100 per cent was found in comparable dark-grown seedlings treated with GA. Growth active zones were found at Rf 0.6 to 0.9 and at Rf 0.0 to 0.3. Both of these zones were found only in seedlings which had been previously treated with GA.

Eluates of paper chromatograms containing extracts of GA-treated seedlings were found to be active in nullifying the red-light inhibition of growth of intact Alaska pea seedlings. Thus, the same zones of chromatograms which had shown activity in the oat first-internode assay were also effective in promoting the growth of pea seedlings whose growth had been inhibited by exposure to red light. The growth substances isolated from seedlings treated with GA are therefore substances which possess "gibberellin-like" activity as well as auxin activity. These results are interpreted to mean that GA may induce the formation of a substance(s) which is active in both straight-growth assays and in the reversal of the red-light inhibition of plant growth.

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## INTRODUCTION

It has been suggested that the growth responses elicited by gibberellic acid (GA) are the result of an interaction between the GA applied to the plant and endogenous auxins or growth factors (2, 6). Brian and Hemming (2) have proposed three main possible modes of action. The first concerns a combination of GA with endogenous auxin (or auxins) to form a more active compound. The second proposal is that GA induces an increase in the effective levels of endogenous auxin, while the third suggestion envisages a neutralization by GA of a growth inhibitor or an inhibitory system that would otherwise keep the growth rate at a level below that potentially possible.

The work reported here was initiated to subject the above hypotheses to experimental approaches involving both qualitative and quantitative endogenous auxin and growth-factor analyses. Commonly utilized growth-substance techniques were used to determine the influence of red light, darkness, and GA on the internal growth-substance levels of seedlings of the Alaska pea. This was considered a reasonably direct approach to the problem, since red light inhibits growth and GA overcomes such inhibition.

RESPONSES OF PEA SEEDLINGS TO RED LIGHT AND  
TO GIBBERELIC ACID

For the first two series of extractions (Experiments I and II) seed of Alaska pea was sown in moist, washed river sand. In the third series (Experiment III) the seed was planted on the surface of moist vermiculite. In all cases, once the seed was planted and germinated the subsequent seedlings were grown in either darkness or in red light at temperatures of 26° to 28° C.

Pea seedlings in red light grow more slowly for the first week after planting than do similar seedlings in total darkness (4, 6). However, seedlings sprayed with GA grow as rapidly as those in the dark (4, 6). Thus, the growth rate of the seedlings inhibited by red light is increased more than that of plants in the dark.

In previous work on this subject (6) the GA was always applied in the form of an aqueous spray and in Experiment II, described below, the same technique was used. In Experiment III the GA treatment was administered by allowing the seed to imbibe a 100 p.p.m. aqueous solution of the compound overnight before planting in vermiculite. The corresponding untreated seed was soaked in a comparable volume of water.

In Experiment II GA was applied as a 10 p.p.m. aqueous solution containing <1.0 per cent Tergitol NPX as a wetting agent. Controls were sprayed with appropriate solutions containing similar quantities of the detergent.

The manipulations connected with spraying and harvesting were performed either in darkness or in dim light from a 40-watt, green fluorescent

lamp which had been wrapped in three layers each of amber and green cellulose acetate sheets, emitting at 546 millimicrons (5).

#### EXTRACTION AND CHROMATOGRAPHIC PROCEDURES

The entire aerial shoots of the seedlings were severed from the cotyledons, quickly frozen at  $-15^{\circ}\text{C}$ ., and kept in complete darkness. The material was frozen further with solid carbon dioxide, and macerated in a Waring Blendor with absolute ethanol. The ethanolic, macerated material was submerged in an excess of ethanol and allowed to stand at  $-4^{\circ}\text{C}$ . for 24 hours. At the end of the extraction period the ethanol extract was filtered and the tissue was washed again with fresh ethanol. These washings were added to the original filtrate and the extracted solids retained for dry weight determinations. (The term "dry weight" refers to weight *after* extraction.)

After filtration the extracts were concentrated by rotation in a flask at  $35^{\circ}$  to  $40^{\circ}\text{C}$ . under reduced pressure. The clear, amber aqueous fraction at pH 4.0 was extracted four times with anhydrous, ethyl ether. The ether extracts therefore contained the acidic, ether-soluble materials, since neutral extractions were not made. Only the acid ether fraction of the original extract was tested for growth-substance activity, while the aqueous residue was discarded.

The ether extracts were concentrated to dryness under reduced pressure at room temperature and were made up to convenient volumes with absolute ethanol prior to application on Whatman No. 1 paper for chromatographic analyses.

One milliliter of the extracts, containing the equivalent of 800 mg. of dry tissue in Experiment I and only half this amount in Experiment II, was applied along a 5.0 cm. starting line. The chromatograms were developed in isopropanol—ammonia—water (80:5:15 v/v/v) to a distance of 25.0 cm., in an ascending direction.

#### BIOASSAY OF CHROMATOGRAMS

The developed chromatograms were divided into ten, equal transverse strips between the starting line and the solvent front. Each of these strips was placed in a test tube with two ml. of 2.0 per cent sucrose+buffer (phosphate-citrate pH 5.0). To each of the tubes, ten 4.0-mm., first-internode sections of *Avena* (var. Brighton) were added and the tubes rotated overnight in darkness (5). Final measurements of the sections were made after 24 hours by placing them, after blotting, on a glass slide in a photographic enlarger, and measuring the magnified ( $\times 3$ ) images with a flexible, plastic rule (1).

The results of the bioassay were plotted in histogram form with the increase in length of the first internodes expressed as percentage increase



over the original 4.0 mm. length. Statistical analyses were carried out employing a system of allowances.

### EXPERIMENTAL RESULTS

#### EXPERIMENT I. CHANGES IN ENDOGENOUS GROWTH SUBSTANCES IN RED LIGHT AND IN DARKNESS

Seedlings were harvested for extraction after 5, 6, 7, 8, 9, 10, 11, and 21 days' growth in red light and in darkness. Those in red light were markedly inhibited up to the seventh day but after this time they obtained a height comparable to dark-grown seedlings.

The chromatograms showed several ultraviolet fluorescent bands, the most characteristic occurring at  $R_f$  0.35. Ehrlich-positive reactions were also noted on several chromatograms. These data are presented in Table I.

Results of the bioassays of the chromatographed extracts are shown in Figure 1. Statistically significant differences are indicated by the open bars.

It can be noted that there are either two, one, or no zones of growth-promoting activity present on the chromatograms. The active zones lie in the region of  $R_f$  0.5 to 0.6 and  $R_f$  0.8 to 0.9. The growth-promoting activity at  $R_f$  0.5 to 0.6 corresponds to the position of an Ehrlich-positive spot on some of the chromatograms and also to the position of an authentic IAA sample concurrently developed on a control strip. However, the areas of growth promotion do not always correspond with Ehrlich-positive areas.

Plants exposed to red light not only grew more slowly but also had less growth substance during the early stages of development. Consistent with this, after 5 and 6 days of growth, on the other hand, there is much more extractable growth substance in dark-grown seedlings. This condition persists until the tenth day at which time the growth-substance contents of plants in dark or in red light are approximately equal. After this time up

TABLE I  
EHRlich-POSITIVE REACTIONS ON PAPER CHROMATOGRAMS OF EXTRACTS OF ALASKA PEA SEEDLINGS GROWN IN RED LIGHT AND IN THE DARK FOR 5 TO 8 DAYS

| Age of seedlings<br>(days)    | Seedlings grown in |          |              |            |
|-------------------------------|--------------------|----------|--------------|------------|
|                               | Red light          |          | Dark         |            |
|                               | $R_f$ value        | Color    | $R_f$ value  | Color      |
| 5                             | —                  | None     | 0.55 to 0.64 | Faint blue |
| 6                             | —                  | None     | 0.50 to 0.66 | Faint blue |
| 7                             | —                  | None     | 0.59 to 0.68 | Faint blue |
| 8                             | —                  | None     | 0.53 to 0.62 | Faint blue |
| Authentic 3-indoleacetic acid | 0.56 to 0.65       | Blue     | —            | —          |
| Authentic tryptophan          | 0.47 to 0.55       | Red-blue | —            | —          |

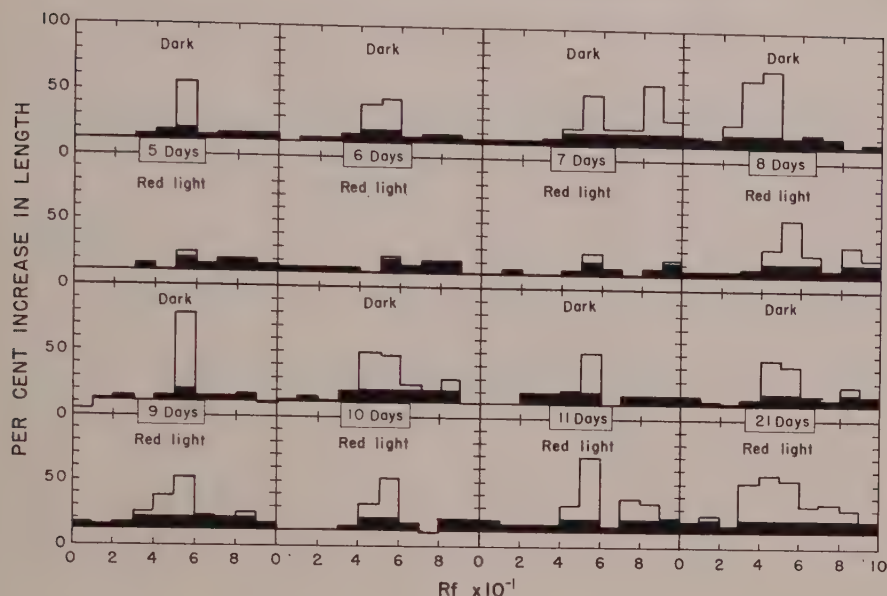


FIGURE 1. Elongation of first internodes of excised oat in eluates from different sectors of a paper chromatogram. Unshaded areas constitute highly significant statistical differences from control.

until the twenty-first day there is more activity in the extracts obtained from seedlings grown in red light.

The qualitative pattern of activity is not so clearly interpreted, for the promoting zone situated proximal to the solvent front is not always present. It shows up markedly in only the seven-day dark and eleven-day red-light samples.

The study of the initial ontogenetic changes in endogenous growth substances revealed one interesting and relevant feature, namely that in the early stages of growth there is less extractable growth substance in plants exposed to light. Consequently the next experiment was to repeat the present one in essentials, and also to investigate the influence of GA on the levels of the endogenous growth substances in seedlings grown in red light and in darkness.

#### EXPERIMENT II. EFFECT OF GA ON ENDOGENOUS GROWTH SUBSTANCES

The previous experiment was repeated up until the tenth day. However, due to an increased temperature of about 5° C. in the culture room, the growth rates were slightly higher than those found previously. The stage of seedling development after a given time in the present experiment does not therefore necessarily correspond to that in Experiment I.

Samples of the seedlings were taken 5, 6, 7, 8, 9, and 10 days after

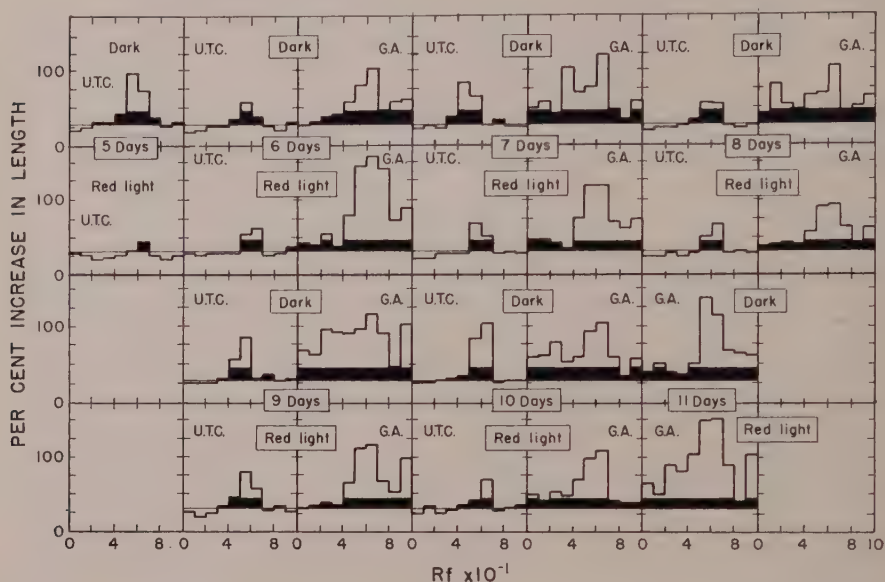


FIGURE 2. Elongation of first internodes of excised oat in eluates from different sectors of a paper chromatogram. Unshaded areas constitute highly significant statistical differences from control.

planting. The results of the bioassays are shown in Figure 2. Again at five days there is a marked difference between the growth-substance levels at  $Rf$  0.5 to 0.7 in dark- and light-grown untreated seedlings. After six days there are approximately equal amounts of growth promotion in extracts of light- and dark-grown plants and this condition persists until the tenth day when there is once again more growth promotion in extracts from dark-grown seedlings.

The areas of the chromatograms which were Ehrlich-positive are recorded in Table II. The positive Ehrlich colors developed on some of the chromatograms were by no means consistently associated with the positions of growth-promoting activity.

Another set of seedlings was grown under similar light and temperature conditions but was sprayed with solutions of GA (10 p.p.m.) on the fifth day. Samples of the GA-treated seedlings were collected on the sixth, seventh, eighth, ninth, tenth, and eleventh day after planting.

The most striking characteristic of seedlings treated with GA, in contrast to untreated seedlings, is the large increase in endogenous growth-substance levels. Both light- and dark-grown seedlings contained much more extractable growth substance if the seedlings had been previously sprayed with GA. Of particular interest is the remarkably high level of growth promotion shown by the extract of the six-day-old, GA-treated,

TABLE II

EHRLICH-POSITIVE REACTIONS ON PAPER CHROMATOGRAMS OF EXTRACTS OF ALASKA  
PEA SEEDLINGS SPRAYED WITH GA SOLUTIONS AND GROWN IN RED LIGHT  
AND IN THE DARK FOR 6 TO 11 DAYS

| Age of seedlings<br>(days)    | Seedlings grown in |             |              |             |
|-------------------------------|--------------------|-------------|--------------|-------------|
|                               | Red light          |             | Dark         |             |
|                               | Rf value           | Color       | Rf value     | Color       |
| 6                             | —                  | None        | 0.42 to 0.53 | Purple-blue |
| 7                             | —                  | None        | 0.41 to 0.50 | Purple-blue |
| 8                             | 0.41 to 0.51       | Faint blue  | 0.45 to 0.55 | Purple-blue |
| 9                             | 0.41 to 0.49       | Faint blue  | 0.40 to 0.47 | Blue        |
| 10                            | 0.40 to 0.51       | Purple-blue | 0.41 to 0.47 | Blue        |
| 11                            | —                  | None        | 0.41 to 0.48 | Blue        |
| Authentic 3-indoleacetic acid | 0.41 to 0.55       | Blue        | —            | —           |

light-grown seedlings. The increase in endogenous growth promoters took place within a 24-hour period after the plants were sprayed with GA.

Qualitative patterns of growth-promoting activity once again present a complex picture. Not only do the growth substances of Rf 0.6 and 0.9 appear, but at least one other peak of activity can be detected between Rf 0.0 to 0.3. This area of growth promotion, as well as the one located at Rf 0.9 was found only in plants which had been treated with GA.

#### EXPERIMENT III. EFFECT OF GA IMBIBED BY PEA SEED ON ENDOGENOUS GROWTH-SUBSTANCE LEVELS IN THE SEEDLING

It was due to the appearance of the histograms in Figure 2 that in the third experiment only one-half (400 mg.) of the previous quantity of plant material was chromatographed. It was apparent that the chromatograms of the extracts from GA-treated plants were overloaded and thus the separation of the several growth substances may have been incomplete.

The seed was soaked overnight, prior to planting, either in water or in an aqueous solution of GA at 100 mg./l. Samples were harvested 4, 5, and 6 days after planting. The results of the bioassays are shown in Figure 3. The over-all effect of GA was to increase very significantly the endogenous growth-substance level. This effect was most pronounced in the five-day-old seedlings. Significant growth promotion occurred in eluates from Rf 0.9 as well as in eluates from Rf 0.4 to 0.6. No significant growth promotion is found in the extract of either four-day-old, untreated or GA-treated seedlings and it is not until the fifth day that there is a discernible difference between the growth substances of GA-treated and untreated tissues. Extracts from six-day-old seedlings show that the GA treatment has obviously increased growth-substance levels, though the qualitative patterns of activity between extracts of dark- and light-grown GA-treated



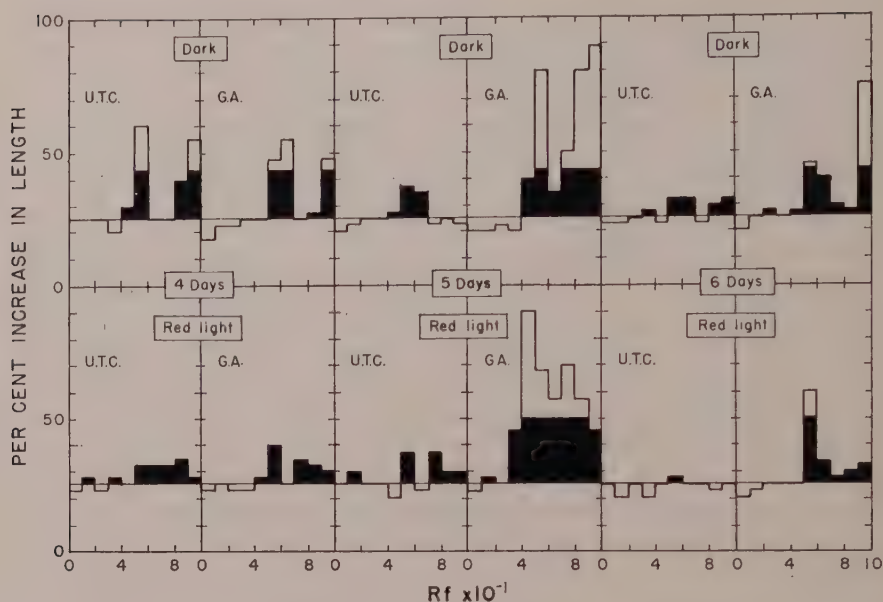


FIGURE 3. Elongation of first internodes of excised oat in eluates from different sectors of a paper chromatogram. Unshaded areas constitute highly significant statistical differences from control.

plants differ. The predominant peak of growth promotion in extracts of six-day-old, dark-grown plants + GA falls at  $R_f$  0.9 to 1.0, whereas in six-day-old light-grown plants + GA it is located at  $R_f$  0.5 to 0.6.

#### EXPERIMENT IV. THE REVERSAL OF RED-LIGHT INHIBITION OF GROWTH IN INTACT PEA SEEDLINGS BY THE GA-INDUCED ENDOGENOUS GROWTH FACTORS

Although the extracts from GA-treated plants were found to contain a considerable quantity of substances which promoted the growth of first internodes of the oat seedling, it was nevertheless questionable whether these substances were in any way related to the growth of intact pea seedlings. Therefore the extracts from GA-treated seedlings were applied to intact pea seedlings under red-light conditions.

The materials at  $R_f$  0.4 to 0.7 and 0.7 to 1.0 on chromatograms of GA-treated seedlings were eluted with 100 ml. of distilled water for this test. One-tenth of a ml. Tergitol NPX was added to the aqueous eluates and these were then sprayed on ten three-day-old pea seedlings grown continuously in red light. Another set of ten seedlings was sprayed with a GA solution (100 p.p.m.), while a third set was sprayed with water containing 0.1 per cent Tergitol NPX solution. Height measurements made before spraying and 24 hours after treatment gave the data presented in Figure 4. Control seedlings which had been sprayed with water + detergent grew an

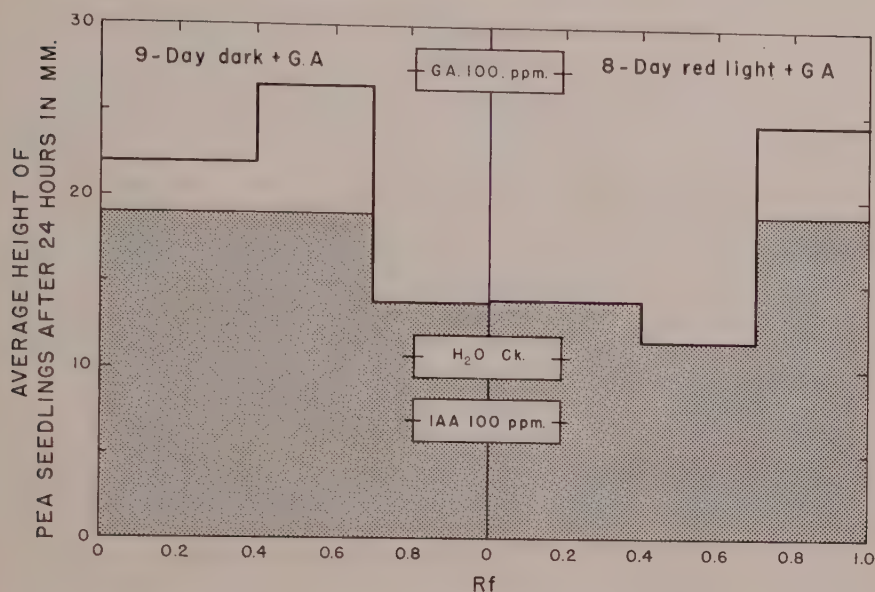


FIGURE 4. Elongation of intact pea seedlings grown in red light after application of sprays with eluates from different sectors of a paper chromatogram. Unshaded areas constitute highly significant statistical differences from control.

average of 10.6 mm. during the 24-hour period, while those sprayed with eluates from Rf 0.4 to 0.7 and Rf 0.7 to 1.0 averaged 26.6 and 24.4 mm., respectively, during the same period of time. Seedlings sprayed with an authentic sample of 100 p.p.m. GA averaged 27.5 mm. The differences between all three treatments and the controls were highly significant statistically.

#### DISCUSSION

The present experiments demonstrate conclusively that GA, whether applied as a spray to seedlings or as a solution to be imbibed by the seed, increases the level of endogenous growth factors which are not only active in promoting the growth of first-internode sections of *Avena* but also increase the growth of intact pea seedlings inhibited by red light. These substances, the formation of which is induced by GA, might be described on the one hand as "auxins" since they are active in a simple auxin assay. On the other hand, they may also be described as "gibberellin-like" since they can overcome the red light-induced inhibition of growth.

Is it possible that the increased growth promotion of oat first-internodes by extracts of GA-treated seedlings can be ascribed to the presence of GA *per se* on the chromatograms? This is a particularly relevant question in the present instance since GA can promote the growth of *Avena* first-internode sections. However, the growth promotion in the range of 1.0

to 100 mg./l. is negligible<sup>4</sup> compared to the very large increases in growth described here. Furthermore, GA could not be detected on the developed chromatograms with a highly sensitive sulfuric acid-ethanol spray followed by scanning under ultraviolet light. This method detects as little as 0.2 microgram of GA. Therefore it follows that the quantity of GA on the chromatograms would be in quantities below this limit and insufficient to account for the growth promotion of first-internode segments.

It may also be argued that the GA applied to the plant synergized some endogenous growth substance or auxin, thereby yielding growth promotion far in excess of what one would expect from GA alone or from auxin alone. This possibility is not ruled out completely but is highly unlikely, since it has been reported by Brian and Hemming (2) that quantities of GA far in excess of 0.2 microgram are required for synergistic action with auxin.

A third possibility is that GA combines with an endogenous auxin to form a complex which has both "auxin" and "gibberellin-like" physiological activity. This has already been suggested by Brian and Hemming (2) and the present results strengthen this hypothesis.

It is also possible that GA induces the formation of a new growth factor with activity both in the straight-growth assay and in the intact seedling.

The present experiments would tend to negate the contention that GA exerts its growth effect by "sparing" auxin since the known auxins will not reverse the red light-induced inhibition of growth of pea seedlings.

Galston (3) has suggested that GA must react with some tissue component before interacting synergistically with IAA, and that GA lowers IAA-oxidase activity by increasing the level of an inhibitor of this enzyme in the tissue. The data presented here are not inconsistent with Galston's findings. It would appear, however, that the primary function of GA is to induce the formation of some highly active substance(s) with both "auxin" and "gibberellin-like" activity rather than to spare endogenous IAA, if IAA is in fact present in these tissues.

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<sup>4</sup> Unpublished data and personal communication with Dr. J. P. Nitsch, Cornell University.

# QUINIC ACID AS A PRECURSOR IN AROMATIC BIOSYNTHESIS IN THE ROSE<sup>1</sup>

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HENRY J. LAURENCOT, JR.

## SUMMARY

Changes in concentration of quinic, succinic and malic acids of Better Times rose petals were studied during aging on the plant. Quinic acid was found to increase in the bloom until the rose was half-opened, after which it decreased. Succinic acid increased until the blooms were half-opened, then remained at a constant level. Malic acid accumulated throughout the aging period. The ratio, malic/quinic, increased steadily with age until the concentration of malic acid in the tissues approached that of quinic acid.

C<sup>14</sup>-labeled quinic acid was biosynthesized in young rose leaves by exposing plants to C<sup>14</sup>O<sub>2</sub> in the light. Aliquots of the labeled quinic acid were fed back to cut roses and allowed to be metabolized for various lengths of time. About 20 per cent of the C<sup>14</sup> fed into the roses was recovered as C<sup>14</sup>O<sub>2</sub> by the time they were senescent. Shikimic acid was the major labeled compound of the nonvolatile organic acids. Tyrosine and phenylalanine represented the bulk of radioactivity of both free and protein amino acids. A major radioactive constituent was found in the neutral fraction, but was not identified.

It is concluded that roses readily metabolize quinic acid and that the relatively high specific activities and high total counts found in shikimic acid, tyrosine and phenylalanine establish that this alicyclic compound can be a precursor in aromatic biosynthesis in the rose.

## INTRODUCTION

The biosynthesis and metabolism of quinic acid are poorly understood in higher plants. Quinic acid and the closely related alicyclic compound, shikimic acid, have both been reported from a number of plant species (1, 8, 9, 10, 12, 13, 15, 29, 30). Depsides, such as chlorogenic acid, which contain quinic acid are of wide occurrence in the plant kingdom. Recently a *p*-coumarylquinic acid was isolated from apples (3, 11, 31).

Shikimic acid has been implicated in polyphenolic biosynthesis (9, 17) and as a precursor of lignin (2, 6), of rutin (25) and of aromatic amino acids (19).

The roles of quinic and shikimic acids have been more clearly established in bacteria than in higher plants. Davis (4) has shown that a number of "ring-free" mutants of *Escherichia coli* requiring tyrosine, phenylalanine, tryptophan and *p*-aminobenzoic acid grew in the presence of shikimic acid alone. Further studies with mutants of *E. coli* and *Neurospora* showed that shikimic, 5-dehydroshikimic and 5-dehydroquinic acids are obligatory intermediates in aromatic biosynthesis (4). Microbial growth response to exogenous quinic acid has been reported in secondary mutants

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of *Aerobacter* (5) and a mutant of *Neurospora* (7). Mitsuhashi and Davis (21) have demonstrated in *Aerobacter* a DPN-linked quinic dehydrogenase which converts quinic to 5-dehydroquinic acid, but the enzyme is not widely distributed in microorganisms. Davis (4, 21) has concluded that quinic acid is not directly involved in aromatic biosynthesis in microorganisms, and that where a requirement has been shown to exist, it is due to an adventitious quinic dehydrogenase.

5-Dehydroquinase (20) and 5-dehydroshikimic reductase (32) have been demonstrated in both spinach and pea plants, but no quinic dehydrogenase has been detected (21).

Hulme and Woollorton (14, 15) have recently made a careful study of the behavior of quinic, shikimic and other nonvolatile organic acids of apples during storage and during development on the tree. During storage both acids increase for a time, and then decrease, the peak concentration of quinic acid preceding that of shikimic acid. The authors suggest that shikimic acid may arise from quinic acid.

The unusually large concentration of *l*-quinic acid in roses (28) prompted a study of the changes in this acid in rose petals during the natural aging process. Results of this experiment led to the biosynthesis of  $C^{14}$ -labeled quinic acid and to studies on its metabolic implications in aromatic biosynthesis in the rose. A preliminary report of this work has been published elsewhere (27).

#### MATERIALS AND METHODS

*Changes during senescence.* For the determination of changes in concentration of quinic acid in Better Times roses (*Rosa* sp.), blooms were cut from plants at four different stages of development. These stages ranged from tight buds (pre-commercial cutting stage) to senescent blooms. Five replicates were analyzed for each stage. Petals were removed and immediately homogenized in 80 per cent ethanol as described previously (26). The ethanolic extract was passed through hydrogen-form Dowex 50-X8 to remove amino acids and then was passed through a 10-cm. bed of acetate-form Dowex 1-X10, 200 to 400 mesh. Nonvolatile organic acids were removed from the Dowex 1 column by gradient elution using a modification of the methods of Palmer (22) and of Hulme and Woollorton (15). The gradient elution was begun using 2.5*N* acetic acid, collecting 100 2-ml. fractions. The eluting liquid was then changed to 6*N* formic acid and continued for an additional 50 tubes.

*Biosynthesis of l-quinic- $C^{14}$  acid.*  $C^{14}$ -labeled quinic acid was biosynthesized at two different times using young, actively-growing rose plants of the variety Redbird, a sport of Better Times. The biosyntheses were carried out with the apparatus shown in Figure 1.  $C^{14}O_2$  was generated by reacting  $BaC^{14}O_3$  and 10 per cent perchloric acid, and was introduced under slight vacuum into a 19-liter bell jar containing rose plants. Incandescent and

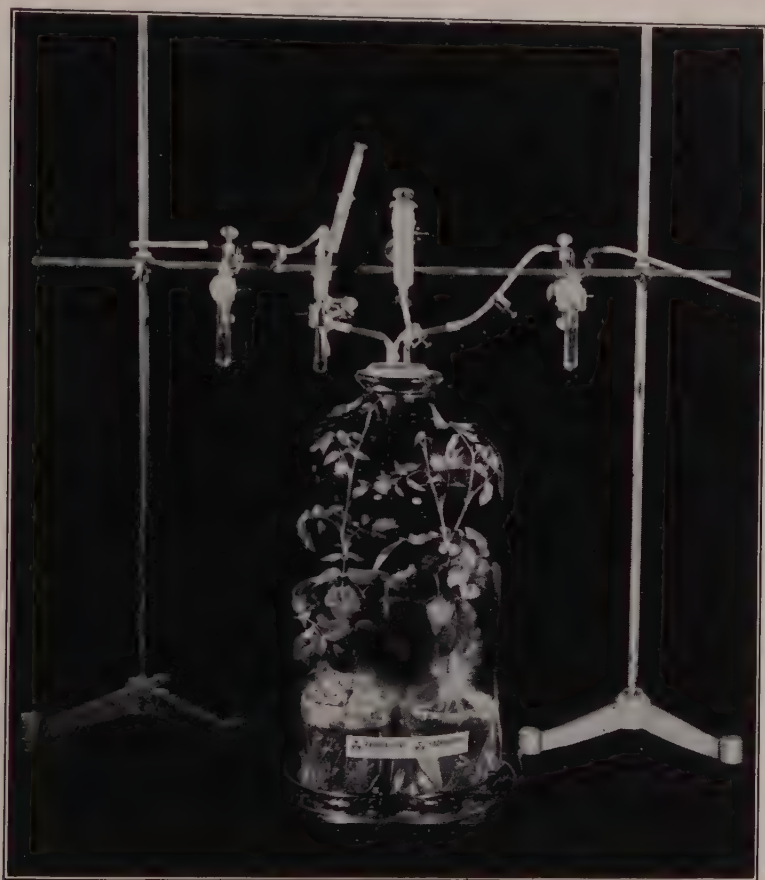


FIGURE 1. Apparatus used for biosynthesis of  $C^{14}$ -labeled quinic acid.

fluorescent lights (approximately 500 f.c. at the surface of the bell jar) were supplied to the plants to promote photosynthetic  $CO_2$  fixation. The temperature never exceeded  $26^\circ C$ . A 20-ml. syringe allowed for aliquots of air to be removed for semi-quantitative  $C^{14}O_2$  analysis. The syringe was pumped several times to agitate the air within the bell jar, and then a 20-ml. aliquot was removed. This was injected into a centrifuge tube containing NaOH and fitted with a rubber serum cap pierced with two needles, one for injection and one to allow the  $CO_2$ -free air to escape.

Barium chloride was added to precipitate  $BaCO_3$ . The tube was centrifuged, the precipitate was washed once with ethanol and an aliquot was counted. At the end of 24 hours, the remaining  $C^{14}O_2$  was flushed out and trapped in NaOH. The bell jar was then recharged with  $C^{14}O_2$ . This was repeated each day for 5 days. At the end of 7 days, leaves were harvested, selecting only those giving an activity of 15,000 c.p.m. or more with a laboratory monitor at the leaf surface. The leaves were ground and ex-

tracted in 80 per cent ethanol. The ethanolic extract was passed through hydrogen-form Dowex 50-X8 to remove amino acids and then through acetate-form Dowex 1-X10, 200 to 400 mesh. The organic acids were removed from the resin by gradient elution with 2.5*N* acetic acid.

The tubes were dried under a stream of air and the contents of the tubes were titrated with NaOH. The shikimic acid and quinic acid eluates were combined separately. The acids were passed through hydrogen-form Dowex 50 to remove sodium and methyl red indicator, and then through formate-form Dowex 1 to take up the acid and brom cresol green indicator. The Dowex 1 resin was eluted with 1*N* formic acid, removing the organic acid but not the indicator. The eluates were taken to dryness. The first biosynthesis yielded about 200 mg. of quinic acid having a specific activity of 11,700 c.p.m./mg. (as counted with a gas-flow Geiger-Mueller tube with a micro-mil window). The second biosynthesis yielded about 500 mg. of quinic acid having a specific activity of 77,200 c.p.m./mg. and about 10 mg. of shikimic acid (specific activity of about 10,000 c.p.m./mg.). The quinic acid was assayed for purity by paper chromatography.

*Feeding experiments.* Two separate feeding experiments with  $C^{14}$ -labeled quinic acid were carried out with young blooms of Better Times roses. The stems were cut to a length of 7 cm. and were placed in small vials containing 0.25 ml. (10 mg.) of quinic acid solution adjusted to pH 6 with NaOH. Thus, in the first experiment, each rose was allowed to absorb 10 mg. of the labeled acid representing 117,000 c.p.m. and in the second experiment, a total of 772,000 c.p.m. After the solution was absorbed, a small quantity of water was pipetted into each vial. When this was absorbed, the roses were transferred to small beakers of water.

The amount of carbon dioxide evolved and its  $C^{14}$  content were measured on a duplicate set of roses placed in a small desiccator. Carbon dioxide-free air was pulled through the container and the contents swept into a trap containing standard  $Ba(OH)_2$  solution. The  $Ba(OH)_2$  trap was removed at intervals, the  $BaCO_3$  was centrifuged, washed with water and recentrifuged. The combined supernatants were then titrated with standard acid using a phenolphthalein end point. The  $BaCO_3$  was suspended in ethanol, and aliquots were placed on planchets and counted.

In the first experiment, two blooms each were harvested at 5, 23, 45 and 71 hours. In the second experiment, two blooms were harvested at 2.5 and 91 hours.

Petals and stem plus receptacle portions were killed in separate containers of boiling 80 per cent ethanol. The tissues were ground with a VirTis "45" homogenizer and extracted as described previously (24). The amino acids were taken up on hydrogen-form Dowex 50-X8, 200 to 400 mesh, and eluted according to the method of Plaisted (23). The ethanolic extract was then passed through a 10-cm. column of acetate-form Dowex

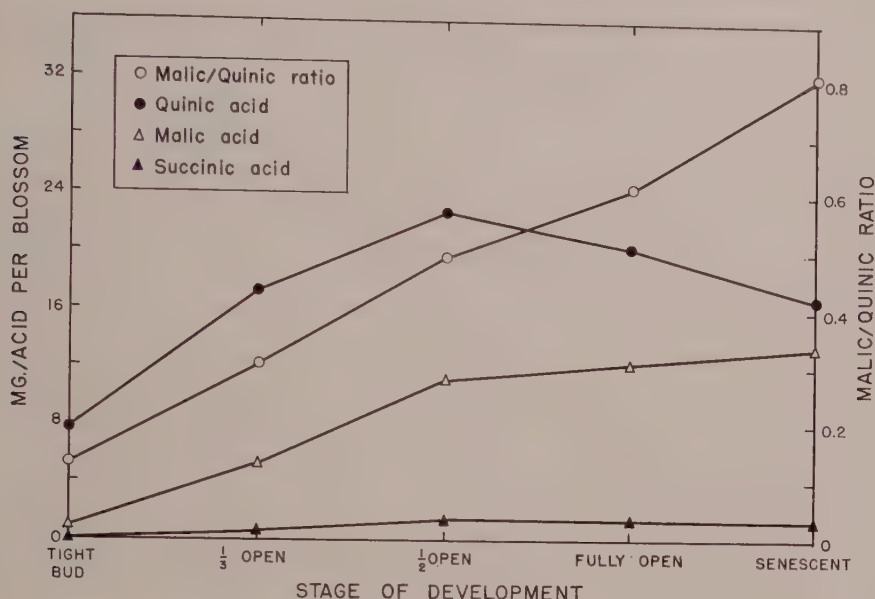


FIGURE 2. Changes in concentration of quinic, malic and succinic acids, and in malic/quinic ratio during natural senescence of petals of Better Times roses.

1-X10, 200 to 400 mesh, to take up organic acids. The organic acids were eluted with 2.5*N* acetic acid followed by 6*N* formic acid as described above. Amino acids were determined quantitatively by the two-dimensional paper chromatographic procedure of Porter *et al.* (24). Titration of the organic acids and determination of radioactivity in the amino and organic acids followed an earlier procedure (26) with the exception that radioactivity on one-dimensional paper chromatograms was determined by eluting 2-cm. segments with 50 per cent ethanol; the eluates were dried on planchets, and counted with a gas-flow Geiger-Mueller tube with a micro-mil window.

Autoradiograms were prepared by exposing Kodak No-Screen X-Ray film to two-dimensional chromatograms for periods up to 4 weeks.

## RESULTS

*Changes in acids during development of rose petals.* Figure 2 shows the changes in quinic, malic and succinic acids at different stages of natural senescence. The concentration of quinic acid increased rapidly in petals until the bloom was about half-opened, after which it decreased. Malic acid increased throughout, as was shown earlier for cut roses. Succinic acid reached a peak in half-opened roses and then remained at a constant level. The amount of shikimic acid in the tissues was too low for accurate measurement, occurring in concentrations of about 1/50 of that of quinic. The



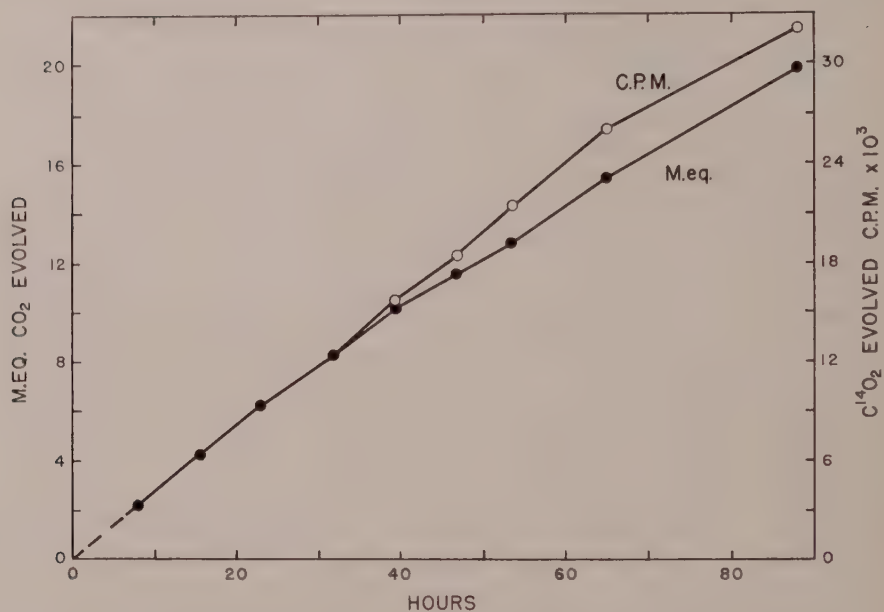


FIGURE 3. Total evolution of CO<sub>2</sub> and radioactivity of C<sup>14</sup>O<sub>2</sub> from roses fed C<sup>14</sup>-labeled quinic acid.

ratio, malic/quinic, increased steadily with age as the concentration of malic acid in the tissues approached that of quinic acid.

*Effect of feeding C<sup>14</sup>-labeled quinic acid to rose petals.* (a) The data for the first feeding experiment are given in Tables I, II and III and in Figure 3. Results show that about 25 per cent of the quinic acid converted to non-volatile organic acids of petals (Table I) was incorporated into shikimic acid at each stage of senescence. A somewhat higher percentage was found in malic acid throughout, while the amount in fumaric acid increased with age. In general, the specific activity data suggest that shikimic acid was the dominant labeled intermediate. In stem and receptacle tissues (Table I), labeled shikimate represented as much as 50 per cent of the total radioactivity of the organic acid pool. The remaining radioactivity was found primarily in malate. On a specific activity basis, most of the organic acids detected had reached about the same level at senescence (71 hours).

About 45 per cent of the total C<sup>14</sup> incorporated into free amino acids showed up in the aromatic compounds tyrosine and phenylalanine (Table II). The remaining activity was detected in threonine, serine, asparagine, glutamine and histidine. C<sup>14</sup> was also present in protein amino acids, in humin and in the sugar fraction (Table III). No separations of these fractions were attempted in the first experiment. Of the total C<sup>14</sup> assimilated

and present in petals after 71 hours, about 26 per cent was represented by shikimate, phenylalanine and tyrosine.

The amount of  $\text{CO}_2$  evolved and its radioactivity are shown in Figure 3. About 20 per cent of the  $\text{C}^{14}$ -labeled quinic acid fed into the tissues was metabolized to  $\text{C}^{14}\text{O}_2$  in 71 hours.

(b) The data for the second feeding experiments are given in Tables IV, V, VI and VII. As much as 64 per cent of the quinic acid assimilated into the organic acid fraction of petals was recovered as shikimate (Table IV). Malate again represented an important labeled intermediate, especially in senescent roses (91 hours). As in the first experiment, fumarate became

TABLE I

INCORPORATION OF  $\text{C}^{14}$  FROM QUINIC ACID INTO NONVOLATILE ORGANIC ACIDS OF PETAL AND OF STEM AND RECEPTACLE TISSUES (QUINIC ACID = 11,700 C.P.M./MG.)

| Time,<br>hrs. | Organic acid              | C.p.m. |                        | C.p.m./mg. |                        | Radioactivity as<br>% of total in<br>organic acids |                        |
|---------------|---------------------------|--------|------------------------|------------|------------------------|----------------------------------------------------|------------------------|
|               |                           | Petals | Stem and<br>receptacle | Petals     | Stem and<br>receptacle | Petals                                             | Stem and<br>receptacle |
| 5             | Shikimic                  | 279    | 153                    | 414        | 196                    | 24.2                                               | 25.9                   |
|               | Quinic                    | 48,800 | 18,400                 | 1,750      | 868                    | —                                                  | —                      |
|               | Succinic                  | 106    |                        | 209        |                        | 9.2                                                |                        |
|               | Malic                     | 442    | 429                    | 92         | 296                    | 38.3                                               | 72.7                   |
|               | Citric                    | 70     | 8                      | 91         | 27                     | 6.1                                                | 1.4                    |
|               | Fumaric                   | 30     |                        | 254        |                        | 2.6                                                |                        |
|               | $\alpha$ -Ketoglutaric    | 226    |                        | 223        |                        | 19.6                                               |                        |
| 23            | Shikimic                  | 343    | 126                    | 775        | 163                    | 27.5                                               | 42.2                   |
|               | Quinic                    | 49,750 | 18,750                 | 1,630      | 838                    | —                                                  | —                      |
|               | Pyrrolidone<br>carboxylic | 157    |                        | 650        |                        | 12.6                                               |                        |
|               | Succinic                  | 98     |                        | 180        |                        | 7.8                                                |                        |
|               | Malic                     | 524    | 152                    | 82         | 199                    | 41.9                                               | 50.8                   |
|               | Citric                    | 54     | 21                     | 177        | 201                    | 4.3                                                | 7.0                    |
|               | Fumaric                   | 74     |                        | 354        |                        | 5.9                                                |                        |
| 45            | Shikimic                  | 349    | 99                     | 492        | 203                    | 27.3                                               | 49.0                   |
|               | Quinic                    | 53,250 | 17,100                 | 1,863      | 1,066                  | —                                                  | —                      |
|               | Pyrrolidone<br>carboxylic | 119    |                        | 374        |                        | 9.3                                                |                        |
|               | Succinic                  | 78     |                        | 207        |                        | 6.1                                                |                        |
|               | Malic                     | 502    | 67                     | 78         | 158                    | 39.2                                               | 33.2                   |
|               | Citric                    | 70     | 36                     | 127        | 400                    | 5.5                                                | 17.8                   |
|               | Fumaric                   | 122    |                        | 447        |                        | 9.5                                                |                        |
| 71            | $\alpha$ -Ketoglutaric    | 40     |                        | 293        |                        | 3.1                                                |                        |
|               | Shikimic                  | 407    | 114                    | 689        | 252                    | 24.2                                               | 38.2                   |
|               | Quinic                    | 47,565 | 12,450                 | 2,007      | 820                    | —                                                  | —                      |
|               | Succinic                  | 211    | 36                     | 478        | 297                    | 12.6                                               | 12.0                   |
|               | Malic                     | 791    | 82                     | 121        | 212                    | 47.0                                               | 27.4                   |
|               | Citric                    | 46     | 31                     | 252        | 435                    | 2.7                                                | 10.4                   |
|               | Fumaric                   | 227    | 36                     | 813        | 266                    | 13.5                                               | 12.0                   |

TABLE II

INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO FREE AMINO ACIDS OF PETALS AFTER 71 HOURS (QUINIC ACID = 11,700 C.P.M./MG.)

| Amino acid            | C.p.m./blossom | Radioactivity as % of total in amino acids |
|-----------------------|----------------|--------------------------------------------|
| Serine + asparagine   | 1,170          | 22.8                                       |
| Glutamine + threonine | 1,050          | 20.5                                       |
| Tyrosine              | 1,090          | 21.3                                       |
| Histidine             | 520            | 10.2                                       |
| Phenylalanine         | 1,290          | 25.2                                       |

more radioactive with increasing time. On a specific activity basis, shikimate was the dominant intermediate, with the exception of fumarate in petals at 91 hours (Table IV). In stem and receptacle tissues (Table IV), labeled shikimate represented as much as 84 per cent of the total incorpora-

TABLE III

TOTAL INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO NONVOLATILE ORGANIC ACIDS, FREE AMINO ACIDS, SUGAR FRACTION, PROTEIN AMINO ACIDS, HUMIN AND  $C^{14}O_2$  OF PETALS AND OF STEM AND RECEPTACLE TISSUES (QUINIC ACID = 11,700 C.P.M./MG.)

| Petals                                      |       |        |          |        |
|---------------------------------------------|-------|--------|----------|--------|
| Fraction                                    | Hours |        |          |        |
|                                             | 5     | 23     | 45       | 71     |
| Organic acids                               | 1,153 | 1,250  | 1,280    | 1,682  |
| Amino acids                                 | 1,430 | 1,790  | 3,640    | 5,120  |
| Sugar fraction                              | 410   | 1,244  | 1,187    | 610    |
| Protein amino acids                         | 462   | 1,520  | 2,075    | 3,025  |
| Humin                                       | 178   | 254    | 308      | 360    |
| Total c.p.m./blossom                        | 3,733 | 6,058  | 8,490    | 10,797 |
| Stems and receptacles                       |       |        |          |        |
| Organic acids                               | 590   | 290    | 202      | 299    |
| Amino acids                                 | 610   | 800    | 1,120    | 2,190  |
| Sugar fraction                              | 430   | 530    | 738      | 613    |
| Protein amino acids                         | 333   | 603    | 585      | 610    |
| Humin                                       | 140   | 252    | 384      | 504    |
| Total c.p.m./blossom                        | 2,109 | 2,484  | 3,029    | 4,216  |
| Petals, stems and receptacles               |       |        |          |        |
| $C^{14}O_2$ evolved                         | 2,100 | 9,480  | - 17,600 | 26,500 |
| Grand total                                 | 7,942 | 18,022 | 29,119   | 41,513 |
| Grand total as % of total quinic acid added | 6.8   | 15.4   | 24.8     | 35.4   |

TABLE IV

INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO NONVOLATILE ORGANIC ACIDS OF PETAL AND OF STEM AND RECEPTACLE TISSUES (QUINIC ACID = 77,200 C.P.M./MG.)

| Time,<br>hrs. | Organic acid           | C.p.m.  |                             | C.p.m./mg. |                             | Radioactivity as<br>% of total in<br>organic acids |                             |
|---------------|------------------------|---------|-----------------------------|------------|-----------------------------|----------------------------------------------------|-----------------------------|
|               |                        | Petals  | Stem and<br>recepta-<br>cle | Petals     | Stem and<br>recepta-<br>cle | Petals                                             | Stem and<br>recepta-<br>cle |
| 2.5           | Shikimic               | 2,690   | 1,565                       | 3,600      | 1,815                       | 63.9                                               | 84.4                        |
|               | Quinic                 | 419,000 | 196,500                     | 19,950     | 10,560                      | —                                                  | —                           |
|               | Succinic               | 108     |                             | 606        |                             | 2.6                                                |                             |
|               | Malic                  | 650     | 213                         | 107        | 156                         | 15.4                                               | 11.5                        |
|               | Citric                 | 255     | 20                          | 440        | 167                         | 6.0                                                | 1.1                         |
|               | Fumaric                | 210     | 18                          | 525        | 117                         | 5.0                                                | 1.0                         |
|               | $\alpha$ -Ketoglutaric | 298     |                             | 106        |                             | 7.1                                                |                             |
|               | Unknown                |         | 37                          |            | —                           |                                                    | 2.0                         |
| 91            | Shikimic               | 2,890   | 358                         | 9,480      | 2,570                       | 32.9                                               | 45.2                        |
|               | Quinic                 | 159,500 | 30,700                      | 19,200     | 6,560                       | —                                                  | —                           |
|               | Succinic               |         | 60                          |            | 2,000                       |                                                    | 7.6                         |
|               | Malic                  | 3,440   | 218                         | 600        | 637                         | 39.2                                               | 27.5                        |
|               | Citric                 | 530     |                             | 2,650      |                             | 6.0                                                |                             |
|               | Fumaric                | 1,920   |                             | 9,580      |                             | 21.9                                               |                             |
|               | Unknown                |         | 156                         |            | —                           |                                                    | 19.7                        |

TABLE V

INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO FREE AMINO ACIDS OF PETAL AND OF STEM AND RECEPTACLE TISSUES (QUINIC ACID = 77,200 C.P.M./MG.)

| Time,<br>hrs. | Amino acid             | C.p.m. |                             | C.p.m./mg. |                             | Radioactivity as<br>% of total in<br>amino acids |                             |
|---------------|------------------------|--------|-----------------------------|------------|-----------------------------|--------------------------------------------------|-----------------------------|
|               |                        | Petals | Stem and<br>recepta-<br>cle | Petals     | Stem and<br>recepta-<br>cle | Petals                                           | Stem and<br>recepta-<br>cle |
| 2.5           | Aspartic               | 495    |                             | —          |                             | 18.7                                             |                             |
|               | Glutamine              | 83     |                             | 274        |                             | 3.1                                              |                             |
|               | Serine                 | 31     |                             | 104        |                             | 1.2                                              |                             |
|               | Valine                 | 60     |                             | 1,278      |                             | 2.3                                              |                             |
|               | Tyrosine               | 1,104  |                             | 16,740     |                             | 41.7                                             |                             |
|               | $\gamma$ -Aminobutyric | 30     |                             | 238        |                             | 1.1                                              |                             |
|               | Phenylalanine          | 845    |                             | 28,140     |                             | 31.9                                             |                             |
| 91            | Aspartic               | 259    | 335                         | —          | —                           | 0.8                                              | 7.0                         |
|               | Glutamic               | 433    |                             | 1,585      |                             | 1.4                                              |                             |
|               | Glutamine              | 87     |                             | 60         |                             | 0.3                                              |                             |
|               | Asparagine             | 3,810  | 1,295                       | 205        | —                           | 12.7                                             | 27.0                        |
|               | Serine                 | 259    |                             | 435        |                             | 0.8                                              |                             |
|               | Threonine              | 427    | 105                         | 690        |                             | 1.4                                              | 2.2                         |
|               | Alanine                | 85     |                             | 795        | —                           | 0.3                                              |                             |
|               | Tyrosine               | 4,360  |                             | 5,370      | —                           | 14.6                                             |                             |
|               | $\gamma$ -Aminobutyric | 270    | 130                         | 2,125      | —                           | 0.9                                              | 2.7                         |
|               | Phenylalanine          | 19,930 | 1,680                       | 5,370      | —                           | 66.8                                             | 35.1                        |



TABLE VI  
INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO PROTEIN AMINO ACIDS OF PETAL AND  
OF STEM AND RECEPTACLE TISSUES AFTER 91 HOURS  
(QUINIC ACID = 77,200 C.P.M./MG.)

| Tissue                   | Amino acid    | C.p.m./blossom | C.p.m./mg. | Radioactivity as<br>% of total in<br>protein amino<br>acids |
|--------------------------|---------------|----------------|------------|-------------------------------------------------------------|
| Petals                   | Aspartic      | 120            | 110        | 3.4                                                         |
|                          | Glutamic      | 46             | 21         | 1.3                                                         |
|                          | Serine        | 229            | 236        | 6.4                                                         |
|                          | Tyrosine      | 1,304          | 1,955      | 36.7                                                        |
|                          | Phenylalanine | 1,852          | 1,726      | 52.2                                                        |
| Stems and<br>receptacles | Aspartic      | 117            | 184        | 9.2                                                         |
|                          | Glutamic      | 182            | 217        | 14.3                                                        |
|                          | Tyrosine      | 366            | 953        | 28.8                                                        |
|                          | Phenylalanine | 575            | 1,337      | 45.2                                                        |
|                          | Histidine     | 32             | 49         | 2.5                                                         |

TABLE VII  
TOTAL INCORPORATION OF  $C^{14}$  FROM QUINIC ACID INTO NONVOLATILE ORGANIC ACIDS,  
FREE AMINO ACIDS, SUGAR FRACTION, PROTEIN AMINO ACIDS, HUMIN AND  $C^{14}O_2$   
OF PETAL AND OF STEM AND RECEPTACLE TISSUES  
(QUINIC ACID = 77,200 C.P.M./MG.)

| Petals                                         |        |         |
|------------------------------------------------|--------|---------|
| Fraction                                       | Hours  |         |
|                                                | 2.5    | 91      |
| Organic acids                                  | 4,211  | 8,780   |
| Amino acids                                    | 2,648  | 29,920  |
| Sugar fraction                                 | 7,325  | 27,800  |
| Protein amino acids                            | 1,540  | 3,551   |
| Humin                                          | 160    | 1,365   |
| Total c.p.m./blossom                           | 15,884 | 71,416  |
| Stems and receptacles                          |        |         |
| Organic acids                                  | 1,853  | 792     |
| Amino acids                                    | 735    | 4,790   |
| Sugar fraction                                 | 2,800  | 5,285   |
| Protein amino acids                            | 620    | 1,272   |
| Humin                                          | 100    | 1,540   |
| Total c.p.m./blossom                           | 6,108  | 13,679  |
| Petals, stems and receptacles                  |        |         |
| $C^{14}O_2$ evolved                            | 2,900  | 136,650 |
| Grand total                                    | 24,892 | 221,745 |
| Grand total as % of total quinic<br>acid added | 3.2    | 28.7    |

tion into organic acids. The remaining radioactivity was found mostly in malate and in fumarate. Shikimate had the highest specific activity in the organic acid pool.

In petals after 2.5 hours, almost 75 per cent of the label in free amino acids occurred in tyrosine and phenylalanine. This figure was about 80 per cent (Table V) after 91 hours. Remaining activity was well distributed in aspartate, glutamate, asparagine, glutamine, serine, threonine, alanine and  $\gamma$ -aminobutyrate. At 91 hours, the specific activity of tyrosine and phenylalanine was much higher than that of the other amino acids, both compounds being labeled to the same degree. Similar results were shown for stem and receptacle tissues (Table V).

Radioactivity of protein amino acids of petals and of stem and receptacle tissues at 91 hours (Table VI) was associated primarily with phenylalanine and tyrosine, amounting to 90 and 75 per cent, respectively, of the total in this fraction. Label was also present in aspartate, glutamate, serine and histidine.

Two unidentified radioactive compounds were found in the sugar fraction (neutral fraction) of senescent petals. The compounds had  $R_f$  values in *n*-butanol—acetic acid—water (4:1:5 v/v/v) of about 0.55 and 0.85. The high running component was by far the more radioactive of the two and was originally associated with the yellow flavanoids, quercetin and isoquercitrin. When re-chromatographed in isopropanol—ammonia—water (80:5:15 v/v/v), however, the radioactive component was separated from the pigments and was located at  $R_f$  0.91. This compound has an ultra-violet absorption spectrum which suggests an aromatic structure. No further characterization has been carried out.

The amount of  $C^{14}O_2$  evolved during respiration and a summary of total  $C^{14}$  incorporated are shown in Table VII.

#### DISCUSSION

The rose plant has been shown to readily metabolize quinic acid. In the second experiment, for example, roses at 2.5 hours contained a total of 39 mg. of quinic acid per blossom (exogenously added and endogenous). After 91 hours, the roses contained 26 mg. per bloom. Thus, about one-third of the total quinic acid was metabolized during aging. This is verified by the data in Table VII.

The relatively high specific activities and high total counts in shikimate, tyrosine and phenylalanine establish their proximity to the primary metabolic pathway of quinic acid. In most respects, it appears that higher plants possess a mechanism for conversion of alicyclic to aromatic acids similar to that elegantly demonstrated by Davis and his co-workers for *E. coli* (4) (Fig. 4). The notable exception is that quinate can act as a primary substrate and that a quinic dehydrogenase may be operative in the

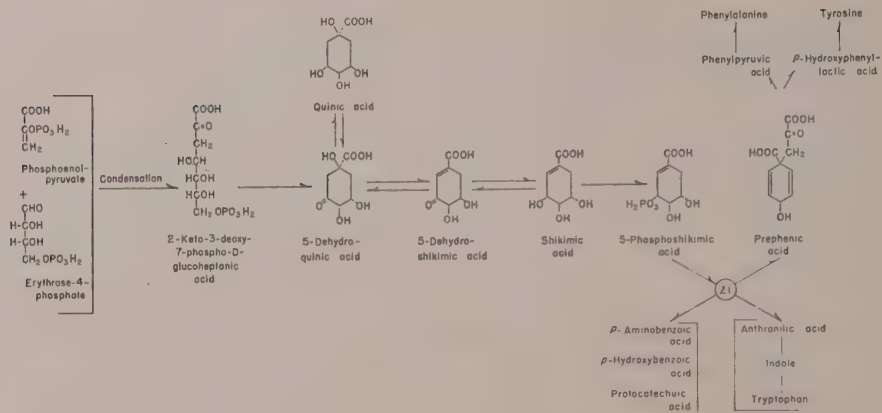


FIGURE 4. Known pathways of biosynthesis of aromatic amino acids in microorganisms. After Davis (4).

rose. However, the high, specific activity of tyrosine and phenylalanine in the blossoms as compared with shikimate after 2.5 hours (Tables IV and V) suggests that, in the rose, shikimate may not be in the direct pathway between quinate and tyrosine and phenylalanine. The relatively higher specific activity of shikimate, as compared with the aromatic amino acids, after 91 hours' incorporation supports this possibility. On the other hand, McCalla and Neish (19) have recently shown that shikimate is an efficient precursor of the aromatic amino acids in *Salvia*. Further experiments now in progress will enable clarification of the role of shikimate in the aromatic pathway.

The distribution of  $C^{14}$  in Krebs cycle acids and in free amino acids may reflect active dark  $CO_2$  fixation, which has been demonstrated in the rose (26). The  $CO_2$  for dark fixation could come from decarboxylation of quinate, by conversion of prephenic to phenylpyruvic acid (16), or by more complete degradation. An alternate pathway for incorporation of label into malate, and thus to other acids, involves the degradation of phenylalanine and tyrosine to malate and acetoacetate (18).

Further experiments are now in progress to detect the enzyme required for the sequential conversion of quinic to shikimic acid via 5-dehydroquinic and 5-dehydroshikimic acids and the metabolism of  $C^{14}$ -labeled quinic acid by other higher plants. The conversion of quinic acid to prephenic and phenylpyruvic acids and the implication of quinic acid in indole biosynthesis are also being investigated.

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# ISOLATION OF RESINS FROM VANILLA BY CHLOROFORM-AMINE EXTRACTION<sup>1</sup>

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## SUMMARY

The resins in vanilla extract can be removed by extraction with chloroform containing 1,3-di-*o*-tolylguanidine. After evaporation of the chloroform, the resins are liberated from their guanidinium salts by treatment of the residue with acetic and hydrochloric acids. They are then recovered by filtration, separated into tetrahydrofuran-soluble and methanol-soluble fractions, and weighed. This procedure may be potentially useful for isolating various other components of plant extracts for quantitative analysis.

## INTRODUCTION

A method has been described for the isolation of resins and other components of vanilla extract (1) which is based in part on the official A.O.A.C. procedure (3, p. 324) for the determination of resins. This involves a tedious distillation or evaporation step and a filtration that is sometimes very slow. A more convenient procedure has now been developed which is based on the observation that vanilla resins are weakly acidic and can be extracted from water-alcohol solutions with chloroform containing a high molecular weight amine. This is similar in some respects to a method proposed for extracting mineral acids from protein hydrolysates (2, 5).

Originally this step was intended to be part of a comprehensive scheme for the isolation of resins, vanillin, amino acids, organic acids and other components of plant extracts prior to quantitative analysis. However, this investigation has now been discontinued so the extraction procedure is being presented here solely as a method for resins.

## MATERIALS AND METHODS

*Reagents.* To prepare solvent A, dissolve 30 grams of 1,3-di-*o*-tolylguanidine (Eastman No. 2169) in chloroform and make the volume up to one liter. To prepare solvent B, dissolve 10 grams of di-*o*-tolylguanidine in chloroform, make the volume up to one liter and add 250 ml. of absolute ethanol. Additional reagents which are required are 0.4*N* HCl, 0.05*N* HCl, glacial acetic acid, absolute methanol and tetrahydrofuran (THF). Commercial tetrahydrofuran contains hydroquinone as a preservative. This should be removed by distillation prior to use. However, it should be noted that peroxides may form on aging. Consequently, samples which give a brown color with acidified KI should not be used or explosions may result on evaporation.

*Procedure.* Use 100 ml. of single strength vanilla extract (4, p. 1266)

<sup>1</sup> This work was supported by a grant from the Flavoring Extract Manufacturers' Association of the United States and the Vanilla Bean Association.

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containing 35 per cent alcohol, or if the sample is a tenfold vanilla<sup>2</sup> dilute a 10-ml. portion to 100 ml. with a 35 per cent (v/v) solution of alcohol in water. Place the sample in a pear-shaped 250-ml. separatory funnel and shake with 20 ml. of solvent A. When the emulsion breaks, drain the lower layer, the interface and a small portion of the upper layer through a plug of glass wool held in a small funnel into a second 250-ml. separatory funnel. This will remove emulsified material which accumulates at the interface. Drain off the lower (chloroform) layer in the second separatory funnel into a beaker. Recombine the upper (aqueous) layers in the first separatory funnel and extract twice more with 25-ml. portions of solvent B, employing the same equipment and technique used in the first extraction.<sup>3</sup> Combine the lower (chloroform) layers in a 250-ml. beaker, add 1 ml. of glacial acetic acid and evaporate the solvent on a hot water bath under a current of clean dry air. Add 1 ml. of glacial acetic acid to the residue and mix thoroughly. Next add 40 ml. of 0.4*N* HCl and stir with a glass rod until the residue has been converted to a finely divided precipitate. Add 40 ml. of water and let the mixture stand at room temperature for a minimum of one hour. Filter out the resin on a medium porosity fritted glass funnel. Wash first with two 5-ml. portions of 0.05*N* HCl, next with two 5-ml. portions of dichloromethane<sup>4</sup> and finally with two 5-ml. portions of 0.05*N* HCl. Air dry the precipitate and attach the funnel containing it to a Fisher Filtrator (catalog No. 9-788), using a tared 50-ml. beaker as a receiver. Wash the residue with 5-ml. portions of warm redistilled tetrahydrofuran until the filtrate comes through colorless. Evaporate the solvent on a hot water bath under an air jet and dry the resin for one hour at 100° C. Weigh and compute the amount of resin soluble in tetrahydrofuran. Treat the residue remaining on the filter with 5-ml. portions of absolute methanol until the filtrate comes through colorless. Evaporate the solvent and dry the residue at 100° C. as described above. Compute the amount of resin soluble in methanol and insoluble in tetrahydrofuran.

## RESULTS

Replicate analyses made on authentic singlefold vanilla extract prepared from Bourbon third beans<sup>5</sup> gave an average value of 0.083 g. of

<sup>2</sup> Single strength vanilla is defined as the solution obtained by extracting 10 grams of cured fruit of *Vanilla planifolia* (Andrews) with aqueous alcohol to make 100 ml. of extract. The water content of the beans is not specified. A vanilla tenfold is obtained by concentrating vanilla singlefold to 10 times its normal strength.

<sup>3</sup> After extraction the upper phase probably contains some of the amino acids, the carbohydrate fraction, and other neutral or positively charged polar compounds.

<sup>4</sup> The filtrate and washings up to this point probably contain weak organic acids, vanillin and other nonpolar aliphatic and aromatic compounds. The precipitate from Tahiti beans contains about 0.04 mg. of material soluble in dichloromethane.

<sup>5</sup> Bourbon third is a trade name for third quality cured vanilla fruit obtained from Madagascar.

TABLE I

COMPARISON OF CHLOROFORM-AMINE AND A.O.A.C. METHODS FOR DETERMINING THE RESIN CONTENT OF VANILLA EXTRACT PREPARED FROM BOURBON THIRD BEANS

| Grams of resin per 100 ml. of extract<br>(CHCl <sub>3</sub> -amine) |                  |       | Grams of resin per 100 ml.<br>of extract (A.O.A.C.) |
|---------------------------------------------------------------------|------------------|-------|-----------------------------------------------------|
| THF-soluble                                                         | Methanol-soluble | Total | Total                                               |
| 0.085                                                               | 0.011            | 0.096 | 0.099                                               |
| 0.085                                                               | 0.012            | 0.097 | 0.093                                               |
| 0.083                                                               | 0.012            | 0.095 | 0.105                                               |
| 0.082                                                               | 0.012            | 0.094 | 0.102                                               |
| 0.081                                                               | 0.008            | 0.089 | 0.102                                               |
| Av. 0.083                                                           | 0.011            | 0.094 | 0.100                                               |

THF-soluble resin per 100 ml. of extract (Table I). An average of 0.011 g. of methanol-soluble resin was also found so that the total soluble resin content of these beans is 0.094 g. per 100 ml. of extract as determined by this method. The coefficient of variation is 3.3 per cent indicating satisfactory reproducibility for a method of this type. The average resin content was about 6 per cent lower than that found by the A.O.A.C. method (3) on this extract. This difference is not significant. Analyses made on extracts of beans obtained from other sources show that the chloroform-amine method gives results comparable to those obtained by the A.O.A.C. method (Table II).

TABLE II

COMPARISON OF CHLOROFORM-AMINE AND A.O.A.C. METHODS FOR ANALYZING VANILLA EXTRACTS FROM VARIOUS SOURCES

| Source of extract | Grams of resin per 100 ml. of extract |          |
|-------------------|---------------------------------------|----------|
|                   | CHCl <sub>3</sub> -amine              | A.O.A.C. |
| Comores beans     | 0.11                                  | 0.11     |
| Dominican beans   | 0.17                                  | 0.15     |
| Guadeloupe beans  | 0.12                                  | 0.15     |
| Tahiti beans      | 0.064                                 | 0.073    |

The resins obtained by the chloroform-amine procedure are easily filtered regardless of whether the vanilla extracts are authentic, or are commercial singlefolds and tenfolds. This is not always the case for resins recovered from commercial vanillas by the A.O.A.C. method.

## DISCUSSION

This procedure is based on the finding that chloroform solutions of high molecular weight amines can be used to extract HCl from aqueous solutions of amino acids (2, 5). The large hydrocarbon groups of the amine prevent it from going into the aqueous phase, while the amine hydrochloride is a



neutral salt that is readily soluble in chloroform. However, in neutral and acid solutions, amino acids bear positive charges and so will not go into the organic phase. This makes possible a fairly clean-cut separation of amino acids from other organic acids and some mineral acids.

Pure vanilla extracts are normally acidic, and the resins they contain are weak acids. Consequently, when shaken with chloroform-amine reagent, the resins accumulate in the organic phase even though they are insoluble in chloroform alone. The alcohol present in the aqueous extract may assist in the transfer by forming a ternary amine-chloroform-ethanol system which may contain traces of water. This is indicated by the fact that the volume of the chloroform layer increases about 20 per cent during extraction. For this reason and also to aid in breaking emulsions, alcohol is a component of the reagent used in making the second and third extractions.

The chloroform solution containing the resin-amine salt is easy to evaporate, and the water-insoluble resin can be regenerated by treating with acid. HCl is preferred for this purpose since the hydrochloride of di-*o*-tolylguanidine is soluble in water, and the acid itself can be removed by evaporation should this prove necessary in the analysis of other components in this fraction. However, for reasons yet unknown it is necessary to add a small amount of acetic acid to the extract in advance of evaporation and to treat the residue with acetic acid before adding the HCl in order to get finely divided resin particles. The resins obtained by this technique are very clean and filter easily. However, the 0.4*N* HCl used to precipitate them must be diluted with water once they are converted to powders, for otherwise the resins from some extracts tend to become gummy on prolonged standing in the stronger acid.

A number of amines were evaluated in this investigation but only di-*o*-tolylguanidine gave completely satisfactory results. Dimethyldodecylamine, a compound used in these laboratories for extracting fluorescent pigments from vanilla, gave very low values for resin content. Triphenylguanidine extracted the resins satisfactorily but the solubility of its hydrochloride in water is so low that some of it would be filtered out with the resins.

The filtrate and the washings from the resin determination probably contain weak organic acids, vanillin and other aromatic compounds as well as many of the fluorescent pigments previously found in vanilla extract by paper chromatography.<sup>6</sup> The supernatant probably contains some of the amino acids, the carbohydrate fraction, and other aqueophilic compounds. Thus, this technique might be potentially useful for separating some of the broad classes of chemical compounds found in plants. Neutral

<sup>6</sup> Burchfield, H. P. and Edward A. Prill. Unpublished data.

or even alkaline solutions might be fractionated by adding a strong acid that would remain predominantly in the aqueous phase where it could suppress the ionization of the weak organic acids, and thus render them extractable. Hydrochloric acid would not be a good candidate for this application since it would accumulate in the organic phase and bind too much of the amine. Phosphoric acid might be a better choice since it is more evenly distributed between the two phases (5). However, the highest degree of efficiency might be obtained by acidifying with a compound containing two acid groups and a positively charged group, since the latter should greatly reduce the amount of the reagent that would be accumulated in the hydrocarbon phase as an amine salt. Possible examples are cysteic acid and diethylamine- $\beta,\beta'$ -disulfonic acid. This might improve the recovery of organic acids but would not be of aid in the determination of vanilla resins since the natural acidity of authentic extract is already high enough, and the addition of strong acids to vanilla causes the resins to precipitate before they can be extracted.

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# PLANT DISEASE CONTROL BY 5-NITROFURAN DERIVATIVES IN RELATION TO CHEMICAL STRUCTURE<sup>1</sup>

ROBERT G. OWENS

## SUMMARY

Control of early blight and late blight of tomato and of powdery mildew of bean by 5-nitrofurans under greenhouse conditions was studied with regard to the influence of substituents in the 2-position of the furan ring. ED<sub>95</sub> values for the more active derivatives ranged from about 50 p.p.m. for 3-(5-nitro-2-furyl)acrolein to 250 p.p.m. for 1-(5-nitro-2-furyl)-2-nitroethylene.

Compounds containing alkylene groups in the 2-position were generally more active than derivatives with simpler substituents such as nitro, aldehyde, cyano or dichloromethyl groups. In the alkylene series the terminal group had a strong influence on toxicity. The order of activity was aldehyde > nitro > cyano > carboxyl. When the terminal carboxyl group was esterified, activity was enhanced in descending order ethyl > methyl > propyl > amyl. Fungitoxicity was suppressed by addition of terminal hydantoin or pyrrolidone rings or by combining two nitrofurans to form 5-nitro-2-furfuryl 5-nitro-2-furoate or bis-(5-nitro-2-furfurylidene)acetone. The data suggest that substituents with a double bond or conjugated double bonds might be useful for improving activity of other potentially effective fungicides.

## INTRODUCTION

In 1944 Dodd and Stillman (3) reported that a nitro group in the 5-position on the furan ring confers antibacterial activity on a variety of furans substituted in the 2-position. Subsequent synthesis and screening resulted in discovery of several nitrofurans useful for treatment of certain diseases in humans (5, 11, 13, 14) and domestic animals (4, 6, 9, 12). Tests against plant pathogens with some of these compounds revealed that a number of the derivatives possess antifungal activity of the same order of magnitude as commercial fungicides. Although the cost of synthesis and the photolability of the 5-nitrofurans make them commercially noncompetitive for use on plants, some potentially important relationships between disease control and substituents in the 2-position were found and are reported here for future reference.

## MATERIALS AND METHODS

Test procedures were essentially those described by McCallan (7) and McCallan and Wellman (8). For powdery mildew tests, bean plants, *Phaseolus vulgaris* L., var. Tendergreen, were grown in the greenhouse in 4-inch pots in the presence of older plants infested with actively sporulating powdery mildew fungus, *Erysiphe polygoni* DC. The plants were sprayed

<sup>1</sup> This work was supported in part by a grant from Norwich Pharmacal Company, Norwich, New York.

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about 5 days after emergence when infection was visible as dark green spots on the foliage. The chemicals were applied in aqueous solutions or suspensions at various dosages ranging from 100 to 1000 p.p.m., or, for dosage-response curves, 25 to 200 p.p.m. Triton X-100 (alkyl aryl poly-ether alcohol)<sup>2</sup> at 0.0117 per cent was employed as a wetting agent. After spraying, the plants were returned to the greenhouse for 3 to 5 days. They were then rated for disease on a scale from 0 for no infection to 10 for infection equivalent to controls. In all tests controls were 90 to 100 per cent covered with mycelium and conidia.

For early and late blight tests, 3-week-old tomato plants (*Lycopersicon esculentum* Mill., var. Bonny Best) in 4-inch pots were sprayed with chemical solutions or suspensions at dosages indicated for powdery mildew. The plants were permitted to dry and then they were inoculated with aqueous suspensions of conidia of the early blight fungus, *Alternaria solani* (Ell. & Mart.) Jones & Grout, or with swarm spores of the late blight fungus, *Phytophthora infestans* (Mont.) de Bary. The inoculated plants were kept in humidity chambers (>95 per cent relative humidity) for 24 hours at 70° and 60° F. for early and late blights, respectively. Counts of disease lesions were made after 3 days for early blight and after 4 days for late blight.

Each treatment was replicated at least 3 times in each test. Results are expressed as percentage disease control in all cases.

## RESULTS

### DOSAGE RESPONSE OF EARLY BLIGHT OF TOMATO TO THE MOST ACTIVE 5-NITROFURANS

As shown in Figure 1, A and B, ED<sub>95</sub> values for early blight control for the most active 5-nitrofurans ranged from about 50 p.p.m. for 3-(5-nitro-2-furyl)acrolein to more than 1000 p.p.m. for some derivatives. The slopes of the dosage-response curves were relatively flat for 3-(5-nitro-2-furyl)acrolein, bis-(5-nitro-2-furfurylidene)acetone, 3-(5-nitro-2-furyl)acrolein oxime and 3-(5-nitro-2-furyl)acrylonitrile (Fig. 1 A), but quite steep for the four compounds shown in Figure 1 B. Preliminary data from studies on the mode of action of some of the above compounds show that 3-(5-nitro-2-furyl)acrolein and 1-(5-nitro-2-furyl)-2-nitroethylene have a similar mode of action although the slopes of the dosage-response curves are different. This suggests that a relationship between slopes of dosage-response curves and the mechanism of action is dubious.

### DISEASE CONTROL BY 5-NITROFURANS IN RELATION TO SUBSTITUENTS IN THE 2-POSITION

All of the compounds were tested initially at 1000 and 400 or at 1000 and 200 p.p.m. Those affording as much as 90 per cent control at 200

<sup>2</sup> Manufactured by Rohm & Haas Company, Philadelphia, Pa.

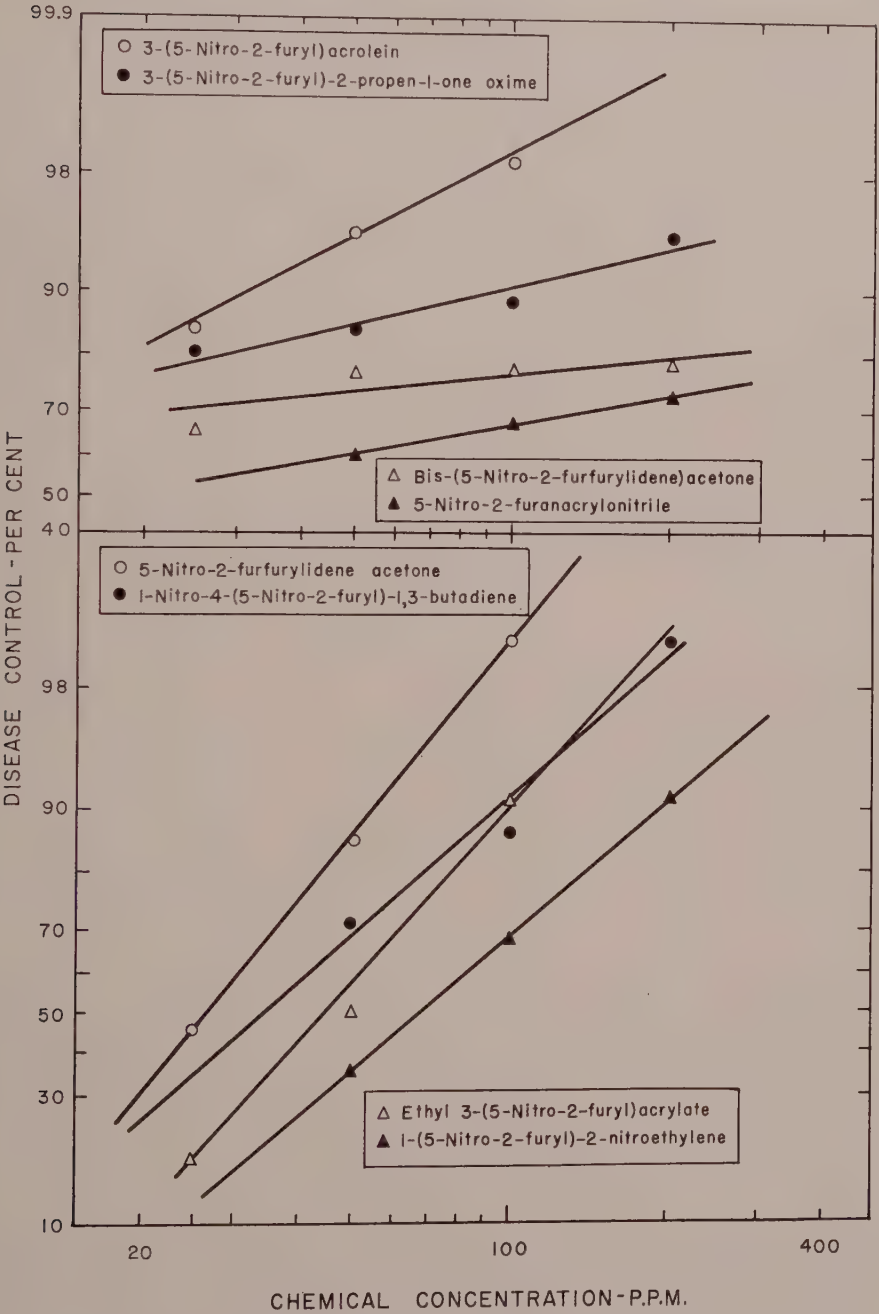
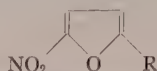


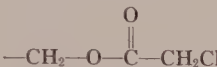
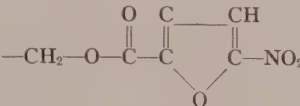
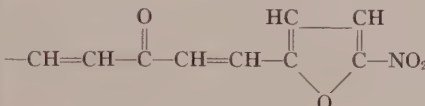
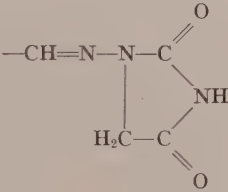
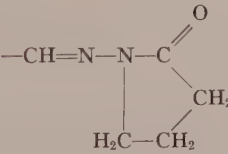
FIGURE 1 (A and B). Dosage-response curves for some of the more active 5-nitro-furan derivatives against early blight disease of tomato in the greenhouse.

TABLE I  
CONTROL OF EARLY BLIGHT OF TOMATO BY 5-NITROFURAN DERIVATIVES  
VARIOUSLY SUBSTITUTED IN THE 2-POSITION



| Name                                         | R                                                                                   | Percentage disease control<br>at chemical concentration<br>(p.p.m.) of |     |     |     |
|----------------------------------------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------------|-----|-----|-----|
|                                              |                                                                                     | 1000                                                                   | 400 | 200 | 100 |
| 2,5-Dinitrofuran                             | —NO <sub>2</sub>                                                                    | 59                                                                     |     | 35  |     |
| 5-Nitrofuraldehyde                           | —CHO                                                                                | 0                                                                      |     |     |     |
| 5-Nitro-2-furonitrile                        | —CN                                                                                 |                                                                        | 85  |     |     |
| 5-Nitro-2-furfurylchloride                   | —CH <sub>2</sub> Cl                                                                 | 0                                                                      |     |     |     |
| 3-(5-Nitro-2-furyl)acrolein                  | —CH=CH—CHO                                                                          |                                                                        |     |     | 98  |
| 3-(5-Nitro-2-furyl)acrylonitrile             | —CH=CH—CN                                                                           |                                                                        |     | 73  | 70  |
| 1-(5-Nitro-2-furyl)-2-nitro-ethylene         | —CH=CH—NO <sub>2</sub>                                                              |                                                                        |     | 92  | 68  |
| 1-(5-Nitro-2-furyl)-2-nitro-1-propene        | $\begin{array}{c} \text{CH}_3 \\   \\ \text{—CH=C—NO}_2 \end{array}$                | 100                                                                    |     | 99  |     |
| 3-(5-Nitro-2-furyl)acrylic acid              | —CH=C—COOH                                                                          |                                                                        | 56  |     |     |
| Methyl 3-(5-nitro-2-furyl)-acrylate          | $\begin{array}{c} \text{O} \\    \\ \text{—CH=CH—C—O—CH}_3 \end{array}$             |                                                                        | 94  |     |     |
| Ethyl 3-(5-nitro-2-furyl)-acrylate           | $\begin{array}{c} \text{O} \\    \\ \text{—CH=CH—C—O—C}_2\text{H}_5 \end{array}$    |                                                                        |     | 95  | 52  |
| <i>n</i> -Propyl 3-(5-nitro-2-furyl)acrylate | $\begin{array}{c} \text{O} \\    \\ \text{—CH=CH—C—O—C}_3\text{H}_7 \end{array}$    | 100                                                                    |     | 88  |     |
| <i>n</i> -Amyl 3-(5-nitro-2-furyl)-acrylate  | $\begin{array}{c} \text{O} \\    \\ \text{—CH=CH—C—O—C}_5\text{H}_{11} \end{array}$ |                                                                        |     | 67  |     |
| 3-(5-Nitro-2-furyl)acrolein oxime            | —CH=CH—CH=NOH                                                                       |                                                                        |     | 92  | 29  |
| 5-Nitro-2-furfurylidene-acetone              | $\begin{array}{c} \text{O} \\    \\ \text{—CH=CH—C—CH}_3 \end{array}$               |                                                                        |     | 99  |     |
| 1-Nitro-4-(5-nitro-2-furyl)-1,3-butadiene    | —CH=CH—CH=CH—NO <sub>2</sub>                                                        |                                                                        |     | 97  | 88  |
| 5-Nitro-2-furaldehyde acetylhydrazone        | $\begin{array}{c} \text{O} \\    \\ \text{—CH=NNHC—CH}_3 \end{array}$               |                                                                        |     | 97  | 97  |

TABLE I (Continued)

| Name                                                  | R                                                                                  | Percentage disease control<br>at chemical concentration<br>(p.p.m.) of |     |     |     |
|-------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------|-----|-----|-----|
|                                                       |                                                                                    | 1000                                                                   | 400 | 200 | 100 |
| Isopropyl 5-nitro-2-furyl<br>ketone                   |   | 20                                                                     |     | 0   |     |
| 5-Nitro-2-furfuryl chloro-<br>acetate                 |   | 95                                                                     |     | 90  |     |
| 5-Nitro-2-furfuryl 5-nitro-<br>2-furoate              |   |                                                                        |     | 72  | 37  |
| Bis-(5-nitro-2-furfurylidene)-<br>acetone             |   |                                                                        |     | 81  | 80  |
| N-(5-Nitro-2-furfurylidene)-<br>1-aminohydantoin      |   | 45                                                                     |     | 10  |     |
| N-(5-Nitro-2-furfurylidene)-<br>1-amino-2-pyrrolidone |  | 90                                                                     |     | 70  |     |

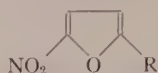
p.p.m. were usually tested also at 100 p.p.m. In instances where control was high at 200 and 100 p.p.m. control values for higher dosages are not reported.

The names, substituents in the 2-position, and the percentage control by the various derivatives are given in Tables I, II and III for early blight, late blight and powdery mildew, respectively.

The simpler derivatives, where the 2-substituent, R, was  $-\text{NO}_2$ ,  $-\text{CHO}$ ,  $-\text{CN}$  or  $-\text{CH}_2\text{Cl}$ , gave relatively poor control of early blight. Where R contained the  $-\text{CH}=\text{CH}-$  moiety, control was invariably increased, but the terminal group was also critical. When the chain terminated in an aldehyde group, activity was high, e.g., 98 per cent control at 100 p.p.m. When a carboxyl group was terminal, control was only 56 per



TABLE II  
CONTROL OF LATE BLIGHT OF TOMATO BY 5-NITROFURAN DERIVATIVES  
VARIOUSLY SUBSTITUTED IN THE 2-POSITION

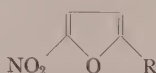


| Name                                      | R                                                                                                                                                                                                                                              | Percentage disease control<br>at chemical concentration<br>(p.p.m.) of |     |     |
|-------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|-----|-----|
|                                           |                                                                                                                                                                                                                                                | 400                                                                    | 200 | 100 |
| 5-Nitrofuraldehyde                        | —CHO                                                                                                                                                                                                                                           | 31                                                                     |     |     |
| 3-(5-Nitro-2-furyl)acrylonitrile          | —CH=CH—CN                                                                                                                                                                                                                                      | 70                                                                     |     | 46  |
| 3-(5-Nitro-2-furyl)acrolein               | —CH=CH—CHO                                                                                                                                                                                                                                     |                                                                        | 99  | 95  |
| 1-(5-Nitro-2-furyl)-2-nitro-ethylene      | —CH=CH—NO <sub>2</sub>                                                                                                                                                                                                                         |                                                                        | 92  | 58  |
| 3-(5-Nitro-2-furyl)acrylic acid           | —CH=CH—COOH                                                                                                                                                                                                                                    | 70                                                                     |     |     |
| Methyl 3-(5-nitro-2-furyl)-acrylate       | $\begin{array}{c} \text{O} \\ \parallel \\ \text{—CH=CH—C—O—CH}_3 \end{array}$                                                                                                                                                                 | 99                                                                     |     |     |
| Ethyl 3-(5-nitro-2-furyl)-acrylate        | $\begin{array}{c} \text{O} \\ \parallel \\ \text{—CH=CH—C—O—C}_2\text{H}_5 \end{array}$                                                                                                                                                        | 94                                                                     |     | 23  |
| 3-(5-Nitro-2-furyl)acrolein oxime         | —CH=CH—CH=NOH                                                                                                                                                                                                                                  |                                                                        |     | 98  |
| 1-Nitro-4-(5-nitro-2-furyl)-1,3-butadiene | —CH=CH—CH=CH—NO <sub>2</sub>                                                                                                                                                                                                                   | 93                                                                     |     | 49  |
| 5-Nitro-2-furfuryl 5-nitro-2-furoate      | $\begin{array}{c} \text{O} \quad \text{HC} \quad \text{CH} \\ \parallel \quad \diagup \quad \diagdown \\ \text{—CH}_2\text{—O—C—C} \quad \text{C—NO}_2 \\ \quad \quad \quad \diagdown \quad \diagup \\ \quad \quad \quad \text{O} \end{array}$ | 93                                                                     |     |     |
| Bis-(5-nitro-2-furfurylidene)-acetone     | $\begin{array}{c} \text{O} \quad \text{HC} \quad \text{CH} \\ \parallel \quad \diagup \quad \diagdown \\ \text{—CH=CH—C—CH=CH—C} \quad \text{C—NO}_2 \\ \quad \quad \quad \diagdown \quad \diagup \\ \quad \quad \quad \text{O} \end{array}$   | 73                                                                     |     |     |

cent at 400 p.p.m. Compounds with terminal nitro or cyano groups were intermediate, with the nitro compounds being somewhat more effective than the nitrile.

Although 3-(5-nitro-2-furyl)acrylic acid gave relatively poor control, esterification of the carboxyl group markedly increased activity. Activity was, in general, dependent upon the length of the carbon chain of the alcohol moiety. Methyl and ethyl derivatives gave a high degree of con-

TABLE III  
CONTROL OF POWDERY MILDEW OF BEAN BY 5-NITROFURAN DERIVATIVES  
VARIOUSLY SUBSTITUTED IN THE 2-POSITION



| Name                                                  | R                                                                                                                                                                                                                                          | Percentage disease control<br>at chemical concentration<br>(p.p.m.) of |     |     |     |
|-------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|-----|-----|-----|
|                                                       |                                                                                                                                                                                                                                            | 1000                                                                   | 400 | 200 | 100 |
| 5-Nitro-2-furfuryl chloride                           | $-\text{CH}_2\text{Cl}$                                                                                                                                                                                                                    | 35                                                                     |     | 0   |     |
| 2,5-Dinitrofuran                                      | $-\text{NO}_2$                                                                                                                                                                                                                             | 85                                                                     |     | 40  |     |
| 5-Nitro-furaldehyde                                   | $-\text{CHO}$                                                                                                                                                                                                                              | 50                                                                     |     | 0   |     |
| (5-Nitro-2-furoyl)hydrazine                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{NH}-\text{NH}_2 \end{array}$                                                                                                                                                    | 5                                                                      |     | 5   |     |
| Isopropyl 5-nitro-2-furyl ketone                      | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}(\text{CH}_3)_2 \end{array}$                                                                                                                                                 | 0                                                                      |     |     |     |
| 5-Nitro-2-furfuryl chloroacetate                      | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}_2-\text{O}-\text{C}-\text{CH}_2\text{Cl} \end{array}$                                                                                                                                | 45                                                                     |     | 5   |     |
| 3-(5-Nitro-2-furyl)acrylonitrile                      | $-\text{CH}=\text{CH}-\text{CN}$                                                                                                                                                                                                           |                                                                        | 80  |     | 30  |
| 3-(5-Nitro-2-furyl)acrolein                           | $-\text{CH}=\text{CH}-\text{CHO}$                                                                                                                                                                                                          |                                                                        | 95  | 50  | 45  |
| 1-(5-Nitro-2-furyl)-2-nitro-ethylene                  | $-\text{CH}=\text{CH}-\text{NO}_2$                                                                                                                                                                                                         |                                                                        | 85  |     | 40  |
| 5-Nitro-2-furaldehyde<br>acetylhydrazone              | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{N}-\text{NH}-\text{C}-\text{CH}_3 \end{array}$                                                                                                                                 |                                                                        | 30  |     | 10  |
| 3-(5-Nitro-2-furyl)acrolein oxime                     | $-\text{CH}=\text{CH}-\text{CH}=\text{NOH}$                                                                                                                                                                                                |                                                                        | 40  |     | 40  |
| 1-Nitro-4-(5-nitro-2-furyl)-<br>1,3-butadiene         | $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{NO}_2$                                                                                                                                                                                     |                                                                        | 30  |     | 5   |
| N-(5-Nitro-2-furfurylidene)-1-<br>aminohydantoin      | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{N}-\text{N}-\text{C} \\   \quad \quad \quad \diagup \\ \text{H}_2\text{C}-\text{C} \quad \text{NH}_2 \\ \quad \quad \quad \parallel \\ \quad \quad \quad \text{O} \end{array}$ | 0                                                                      |     |     |     |
| N-(5-Nitro-2-furfurylidene)-1-<br>amino-2-pyrrolidone | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{N}-\text{N}-\text{C} \\   \quad \quad \quad \diagup \\ \text{H}_2\text{C}-\text{CH}_2 \\ \quad \quad \quad \diagdown \\ \quad \quad \quad \text{CH}_2 \end{array}$             | 15                                                                     |     | 0   |     |

trol, but activity diminished progressively as the length of the carbon chain was increased further, as in the propyl and amyl derivatives.

The increase in activity brought about by the presence of a vinylene moiety in the side chain was further exemplified by 3-(5-nitro-2-furyl)acrolein oxime and 5-nitro-2-furfurylideneacetone. Data from other tests not reported here show that the corresponding analogues, 5-nitro-2-furaldoxime and methyl 5-nitro-2-furyl ketone, are relatively poor foliage protectants; whereas the derivatives reported here are highly active at 200 p.p.m.

Further extension of the side chain of 1-(5-nitro-2-furyl)-2-nitroethylene to form the 1,3-butadiene derivative did not appreciably increase disease control over the 2-nitroethylene derivative. Similarly, no particular advantage was gained by combining two 5-nitrofuran moieties to form 5-nitro-2-furfuryl 5-nitro-2-furoate through an ester linkage or by direct carbon bonding as in bis-(5-nitro-2-furfurylidene)acetone. Side groups terminating in hydantoin or 2-pyrrolidone had no advantage over many of the simpler substituents and even tended to suppress activity. This was found to be particularly true in the case of hydantoin.

In general, the same patterns of control found for early blight in relation to the 2-substituents prevailed in tests on late blight (Table II) and powdery mildew (Table III).

#### DISCUSSION

The present tests demonstrate that changes in the nature of substituents in the 2-position on the furan nucleus strongly affect disease control. Marked improvement in disease control was obtained by lengthening the carbon chain within certain limits, but the nature of the terminal group also had a strong influence on efficacy. Since increase in the length of the carbon chain increases lipophilicity of the molecule, disease control would appear to be associated in some degree with lipid solubility. That this is not the only factor involved, however, is clearly indicated by other studies in which the influence of chemical structure on absorption by fungus spores and inherent toxicity of these compounds was determined (10).

Some of the compounds appear to be fungistatic instead of fungicidal (10), in which case factors such as photolability, water solubility and persistence on the foliage become as important as lipophilicity. These factors are also governed by chemical structure and their relationship to chemical structure will eventually have to be determined before the over-all performance of each compound on foliage can be fully understood.

The nitroethylene group was investigated previously with regard to fungicidal activity and was found to have sufficient activity to warrant a patent (2). Other compounds with a conjugated system of double bonds and carbon chains of various lengths have also been found to possess high activity (1). Substituents such as some of those discussed in this paper

might be of some value in improving fungitoxicity of other potentially effective fungicides.

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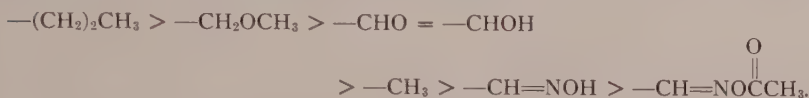


# INFLUENCE OF CHEMICAL STRUCTURE OF 5-NITROFURAN DERIVATIVES ON ABSORPTION BY FUNGUS SPORES AND ON INHERENT TOXICITY<sup>1</sup>

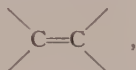
ROBERT G. OWENS AND HELEN M. NOVOTNY

## SUMMARY

The influence of substituents in the 2-position on absorption of twenty-four derivatives of 5-nitrofuran by conidia of *Neurospora sitophila* was determined. The compounds fall into 5 categories, based on the nature of the 2-substituent. Group I is comprised of compounds with relatively simple groups. The order of decreasing absorption in terms of the substituents was found to be



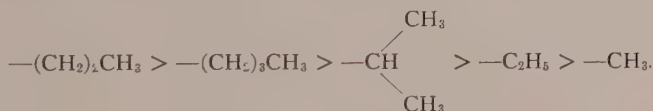
Group II compounds contain a vinylene group,



plus a simple terminal group in the 2-position. The vinylene group tended to increase absorption but its influence was largely dependent upon the terminal group on the chain. Absorption in terms of the influence of the terminal groups was found to be in the following descending order:

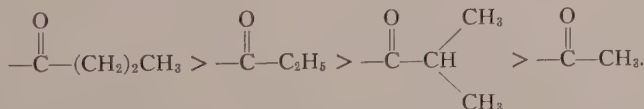


Compounds in Group III are esters of 3-(5-nitro-2-furyl)acrylic acid. Four to 6 times more of the various esters was absorbed than of the free acid. Uptake was strongly influenced by the alcohol moiety:



Group IV contains 2 compounds with terminal rings in the 2-position. With a terminal 2-pyrrolidone ring, uptake was about equivalent to that of some simpler derivatives, but addition of a second ring prevented any detectable uptake.

Group V is a series of ketones. Uptake was influenced by the number of carbon atoms in the chain as follows:



Absorption rate studies show that some of the compounds were taken up in

<sup>1</sup> This work was supported in part by a grant from Norwich Pharmacal Company, Norwich, New York.

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the diluted suspension was then transferred to a microscope slide and mixed with a drop of Fries solution as modified by Ryan *et al.* (16). The slides were incubated in moisture chambers and counts were made when the untreated controls were fully germinated.

After removal of a drop for the germination test, the remaining spores were sedimented in a clinical centrifuge. A portion of the supernatant solution was decanted into a 3-ml. cuvette, 1 cm. in cross-section. The remainder was discarded. The absorbance of the solution was determined immediately with a Beckman Model DU spectrophotometer at the wavelength of maximum absorbance for the compound being tested. The amount of chemical removed from the solution by the spores was calculated from the decrease in absorbance values with reference to appropriate standards.

With all compounds complete absorption spectra in the region between 3500 and 6000 Å was determined before and after exposure to the spores to detect changes in chemical structure due to decomposition. The spectra of the compounds reported here did not change during the test period.

Ten ml. of fresh chemical solution containing 100 µg. of chemical were then added to each centrifuge tube, the spores were resuspended and the procedure as described was repeated for a total of eight times with a 30-minute exposure period each time. Absorption values given in Table I are the sums of the amounts of chemical absorbed in eight exposure periods, unless otherwise stated.

The rate of absorption was determined with the same quantity of spores employed in the experiments described above. Exposure periods were varied from 2 to 300 minutes. The spores were removed from suspension by centrifugation as before.

The chemicals were supplied in crystalline form by Norwich Pharmacal Company. Melting points<sup>2</sup> are given in Table I.

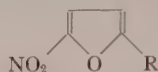
## RESULTS

Preliminary experiments showed that the 5-nitrofurans absorb strongly in both the visible and ultraviolet regions of the spectrum. Although absorbance was greater in the ultraviolet, this region of the spectrum could not be used because leachates from the spores produced high and variable background. Interferences were essentially eliminated when measurements were made with the tungsten lamp. The coefficient of variation in absorbance values for 1-(5-nitro-2-furyl)-2-nitroethylene, 1-(5-nitro-2-furyl)-2-nitro-1-propene and 3-(5-nitro-2-furyl)acrolein in 5 to 8 independent experiments ranged from 1.7 to 4.6 per cent at chemical concentrations between 2 and 10 µg./ml. Thus, within the range of concentrations used,

<sup>2</sup> The melting point data were supplied by Norwich Pharmacal Company, Norwich, New York.



TABLE I  
INFLUENCE OF SUBSTITUENTS ON ABSORPTION OF 5-NITROFURAN DERIVATIVES  
BY NEUROSPORA SITOPHILA CONIDIA



| Name                                                | Melting (or boiling) point, °C. | R                                                                                           | Absorption, $\mu\text{g./g. spore (dry)}$ |
|-----------------------------------------------------|---------------------------------|---------------------------------------------------------------------------------------------|-------------------------------------------|
| <i>Group I</i>                                      |                                 |                                                                                             |                                           |
| 5-Nitro-2-furaldehyde                               | 34-37                           | —CHO                                                                                        | 8,9                                       |
| 5-Nitro-2-furfuryl alcohol                          | 32.5-34.5                       | —CHOH                                                                                       | 8,9                                       |
| 5-Nitro-2-furaldoxime ( <i>anti</i> )               | 159-161                         | —CH=NOH                                                                                     | 6,9                                       |
| 5-Nitro-2-furaldehyde oxime acetate ( <i>anti</i> ) | 107-109                         | —CH=N—O— $\overset{\text{O}}{\parallel}$ C—CH <sub>3</sub>                                  | 3,3                                       |
| Methyl 5-nitro-2-furfuryl ether                     | (104-105/3 mm.)                 | —CH <sub>2</sub> —O—CH <sub>3</sub>                                                         | 10,7                                      |
| 5-Nitro-2-methylfuran                               | 41-43                           | —CH <sub>3</sub>                                                                            | 7,9                                       |
| 5-Nitro-2- <i>n</i> -propylfuran                    | (99-100/4 mm.)                  | —CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>                                            | 32,9                                      |
| <i>Group II</i>                                     |                                 |                                                                                             |                                           |
| 1-(5-Nitro-2-furyl)-2-nitroethylene                 | 143-144                         | —CH=CH—NO <sub>2</sub>                                                                      | 46,3                                      |
| 1-(5-Nitro-2-furyl)-2-nitro-1-propene               | 101-102                         | —CH=C( $\overset{\text{CH}_3}{\diagup}$ )—NO <sub>2</sub>                                   | 16,9                                      |
| 3-(5-Nitro-2-furyl)acrolein                         | 119-120.5                       | —CH=CH— $\overset{\text{O}}{\parallel}$ CH                                                  | 44,9                                      |
| 5-Nitro-2-furanacrylonitrile                        | 111-112                         | —CH=CH—CN                                                                                   | 44,9                                      |
| 3-(5-Nitro-2-furyl)acrolein oxime                   | 175-176                         | —CH=CH—CH=NOH                                                                               | 10,7                                      |
| 3-(5-Nitro-2-furyl)acrylic acid                     | 220-223                         | —CH=CH—COOH                                                                                 | 4,9                                       |
| <i>Group III</i>                                    |                                 |                                                                                             |                                           |
| Methyl 3-(5-nitro-2-furyl)-acrylate                 | 150-151                         | —CH=CH— $\overset{\text{O}}{\parallel}$ C—O—CH <sub>3</sub>                                 | 21,4                                      |
| Ethyl 3-(5-nitro-2-furyl)-acrylate                  | 121-124.5                       | —CH=CH— $\overset{\text{O}}{\parallel}$ C—O—C <sub>2</sub> H <sub>5</sub>                   | 24,9                                      |
| <i>n</i> -Propyl 3-(5-nitro-2-furyl)-acrylate       | 75-76.5                         | —CH=CH— $\overset{\text{O}}{\parallel}$ C—O—(CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub> | 32,5                                      |

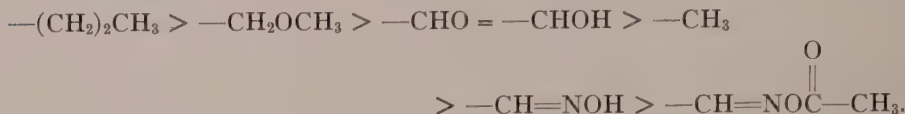
TABLE I (Continued)

| Name                                                                     | Melting (or boiling) point, °C. | R                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Amount absorbed, $\mu\text{g./g.}$ of spores (dry wt.) |
|--------------------------------------------------------------------------|---------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------|
| Propyl 3-(5-nitro-2-furyl)-acrylate                                      | 88-89                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{CH}-\text{C}-\text{O}-\text{CH} \\ \quad \quad \quad \quad \quad \quad \quad \diagup \quad \diagdown \\ \quad \quad \quad \quad \quad \quad \quad \text{CH}_3 \quad \text{CH}_3 \end{array}$                                                                                                                                                                                                                                                                                                                           | 25,800                                                 |
| Butyl 3-(5-nitro-2-furyl)-acrylate                                       | 60-62                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{CH}-\text{C}-\text{O}-(\text{CH}_2)_3\text{CH}_3 \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 28,900                                                 |
| Group IV                                                                 |                                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                        |
| 3-(5-Nitro-2-furfurylidene)-1-amino-2-pyrrolidone                        | 232-232.5                       | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{N}-\text{N}-\text{C} \\ \quad \quad \quad \quad \quad \diagdown \quad \diagup \\ \quad \quad \quad \quad \quad \text{CH}_2 \quad \text{CH}_2 \\ \quad \quad \quad \quad \quad \diagup \quad \diagdown \\ \quad \quad \quad \quad \quad \text{CH}_2-\text{CH}_2 \end{array}$                                                                                                                                                                                                                                            | 7,400                                                  |
| 3-(5-Nitro-2-furfurylidene)-3-amino-5-(N'-piperidylmethyl)-2-oxazolidone | 197-198                         | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{CH}=\text{N}-\text{N}-\text{C} \\ \quad \quad \quad \quad \quad \diagdown \quad \diagup \\ \quad \quad \quad \quad \quad \text{O} \quad \text{O} \\ \quad \quad \quad \quad \quad \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ \text{CH}_2-\text{CH}-\text{CH}_2-\text{N} \quad \text{CH}_2-\text{CH}_2 \quad \text{CH}_2 \\ \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \diagdown \quad \diagup \\ \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \text{CH}_2-\text{CH}_2 \end{array}$ | 0                                                      |
| Group V                                                                  |                                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                        |
| Methyl 5-nitro-2-furyl ketone                                            | 73-5-77                         | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}_3 \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | 11,600                                                 |
| Ethyl 5-nitro-2-furyl ketone                                             | 69-70                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{C}_2\text{H}_5 \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 23,600                                                 |
| Propyl 5-nitro-2-furyl ketone                                            | 59-60                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-(\text{CH}_2)_2\text{CH}_3 \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 56,500                                                 |
| Isopropyl 5-nitro-2-furyl ketone                                         | 57-58                           | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH} \\ \quad \quad \quad \diagup \quad \diagdown \\ \quad \quad \quad \text{CH}_3 \quad \text{CH}_3 \end{array}$                                                                                                                                                                                                                                                                                                                                                                                                        | 20,500                                                 |

differences of the order of 0.5  $\mu\text{g.}/\text{ml.}$  could be measured with 95 per cent confidence<sup>3</sup>.

#### INFLUENCE OF SUBSTITUENTS IN THE 2-POSITION ON ABSORPTION

The relationship between 2-substituents and absorption of the 5-nitrofurans is shown in Table I. For convenience of discussion the compounds are arranged into 5 categories. Group I consists of compounds with relatively simple substituents. The order of decreasing absorption in terms of the 2-substituent was found to be



Extension of the 2-chain with a vinylene group,



tended to increase absorption (Table I). This is apparent when uptake of 3-(5-nitro-2-furyl)acrolein is compared with that of 5-nitro-2-furaldehyde or when 3-(5-nitro-2-furyl)-2-acrolein oxime is compared with 5-nitro-2-furaldoxime. However, the absorption-promoting influence of the vinylene moiety was greatly diminished when the 2-chain terminated in cyano or carboxyl groups compared with nitro or aldehyde groups. Thus, uptake was regulated not only by chain length but, more importantly, by the terminal group on the chain. Absorption in terms of the influence of terminal groups was found to be in the following descending order:

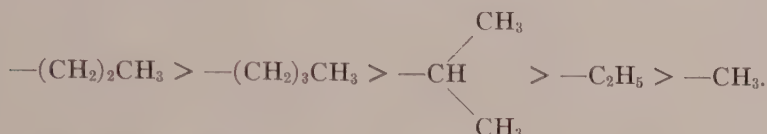


Uptake decreased in the order of increasing hydrophilicity except in the case of 1-(5-nitro-2-furyl)-2-nitro-1-propene. The structural feature of this compound which sets it apart from others in the series is that the 2-chain is branched, one branch consisting of the methyl group and the other of the nitro group. Such terminal branching always seemed to impede uptake, as will be seen from the data on isopropyl derivatives in Groups III and V, Table I.

Compounds in Group III are esters of 3-(5-nitro-2-furyl)acrylic acid. Esterification increased uptake 4- to 6-fold over the free acid, depending upon the composition of the alcohol moiety. Increase in the number of carbon atoms in the alcohol moiety up to 3 increased uptake if they were arranged in a straight chain. Further increase in chain length, as in the

<sup>3</sup> It is a pleasure to acknowledge the services of Dr. Frank Wilcoxon who kindly calculated the coefficients of variation.

butyl ester, resulted in a drop in absorption. A similar decrease was found for the isopropyl ester, which again indicates the impediment of terminal branching in the chain. In descending order, absorption with regard to the alcohol moiety was:



The two compounds in Group IV illustrate the effects of two kinds of rings on uptake. With the 2-pyrrolidone ring, absorption was about equivalent to that of some of the simpler derivatives in Group I. However, addition of a second ring prevented any detectable uptake.

Absorption of the compounds in Group V is not quantitatively comparable to that of compounds in other groups, because they were assayed at twice the initial concentration, e.g., 20 instead of 10  $\mu\text{g./ml.}$  However, comparisons of structural influences within the group can be made, and comparisons of relative effects of chain structure with compounds in the other groups should be valid. It can be seen that uptake increased with increasing number of carbon atoms up to three, as was the case in the acrylate series, Group III. Again, much smaller amounts of the isopropyl derivative were absorbed than of the *n*-propyl derivative.

#### INFLUENCE OF STRUCTURE ON RATE OF ABSORPTION

The influence of structure on rate of uptake of the 5-nitrofuran derivatives was determined by continuous exposure of the spores to 44,500  $\mu\text{g.}$  of chemical per gram of spores over periods of 2 to 300 minutes. The results are given in A-D in Figures 1 and 2.

In agreement with the findings of Miller *et al.* (9) and Burchfield and Storrs (2) for other toxicants, it was found that some of the compounds were taken up in considerable quantities in as little as two minutes. Others apparently penetrated very slowly, requiring up to 10 minutes or more before uptake was sufficient for detection.

The rate of uptake upon continuous exposure did not necessarily follow the same order with regard to 2-substituents as did total uptake in repeated exposures. This was found to be due in part at least to an increase in uptake rate of certain compounds upon repeated exposure to fresh chemical solution, whereas uptake of others dropped off (Fig. 3). This suggests that some compounds disposed the spores to more rapid permeation, possibly by changing the properties of the cytoplasmic membrane.

Another indication of changes in membrane permeability comes from the finding that smaller quantities of some of the compounds, notably 1-(5-nitro-2-furyl)-2-nitro-1-propene and *n*-butyl 3-(5-nitro-2-furyl)acrylate,



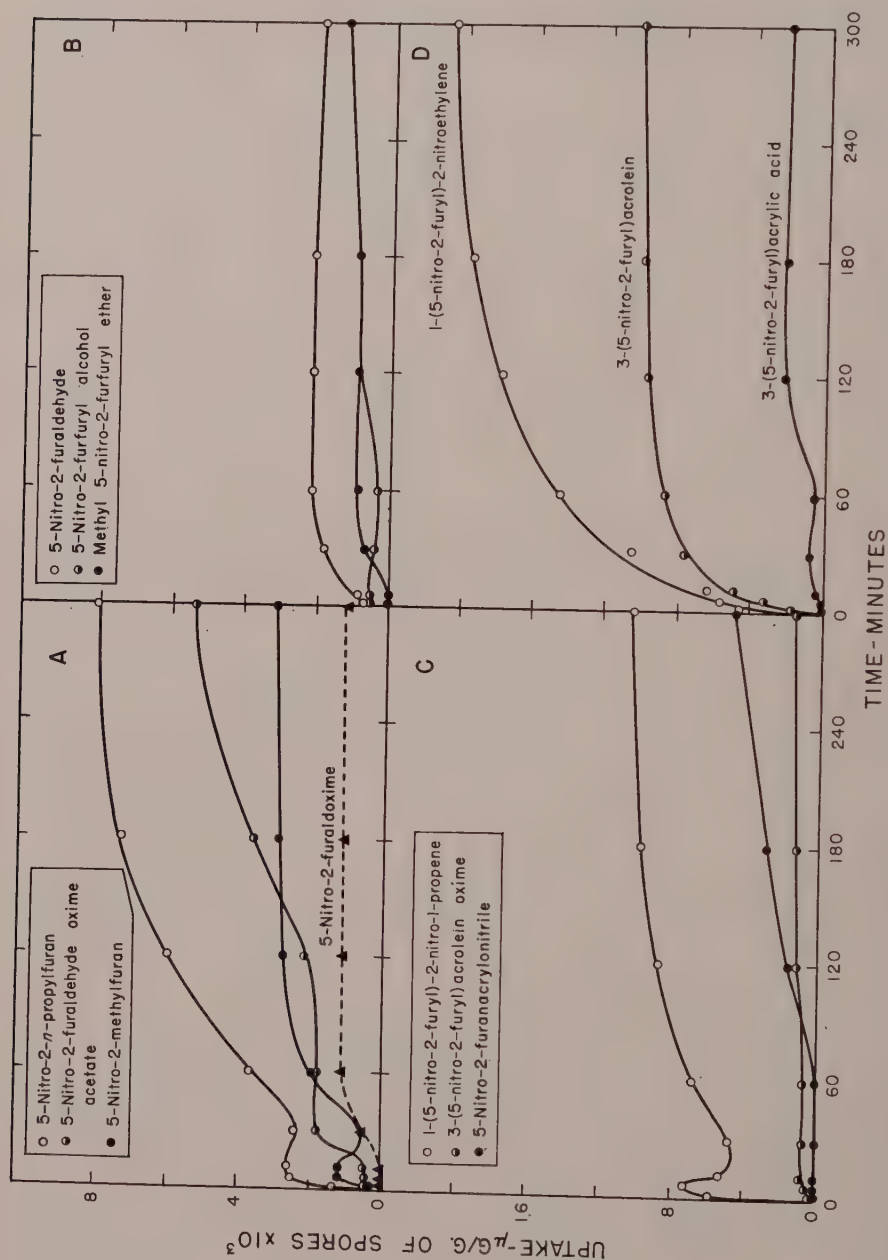


FIGURE 1. A-D. Rate of uptake of various 5-nitrofuran derivatives by conidia of *Neurospora sitophila*.

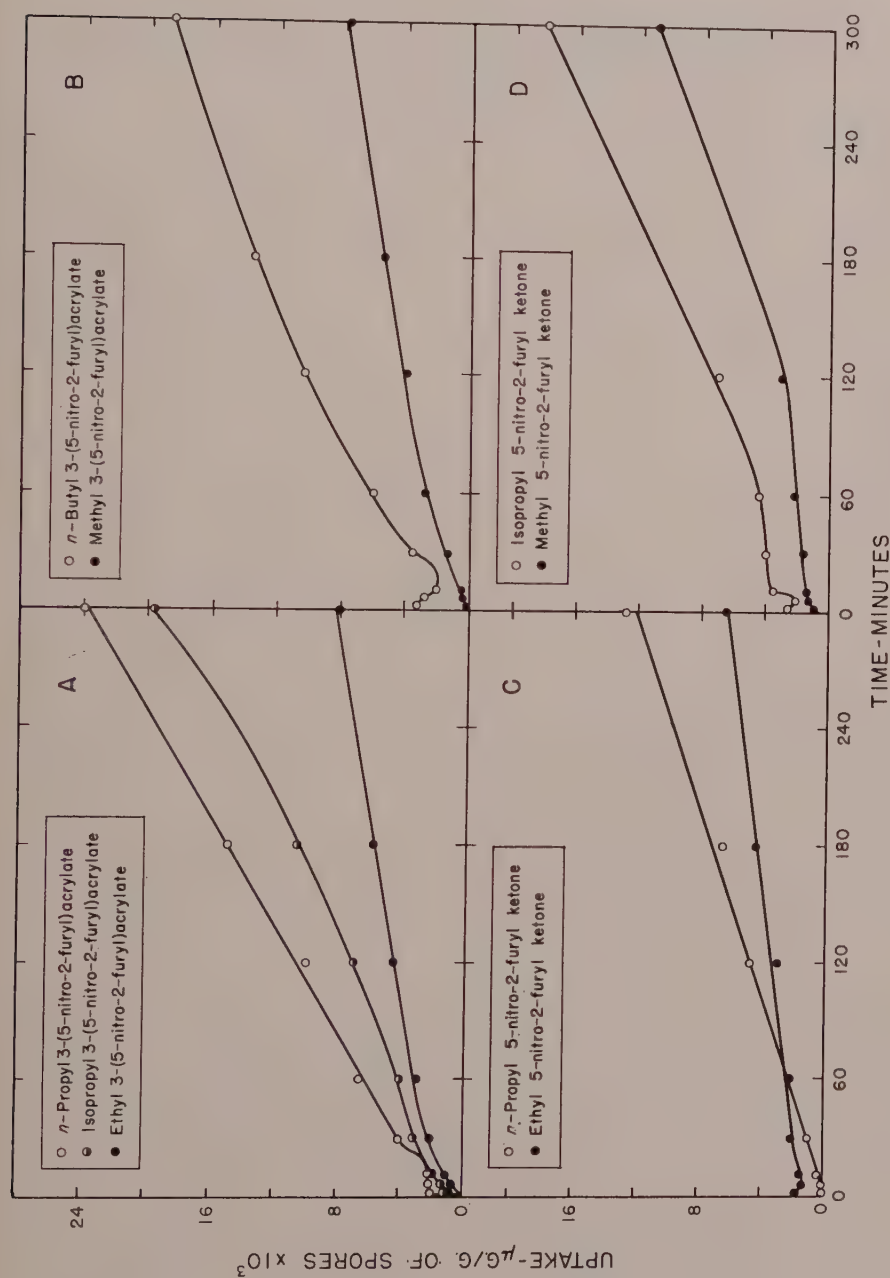


FIGURE 2. A-D. Rate of uptake of various 5-nitrofuran derivatives by conidia of *Neurospora sitophila*.

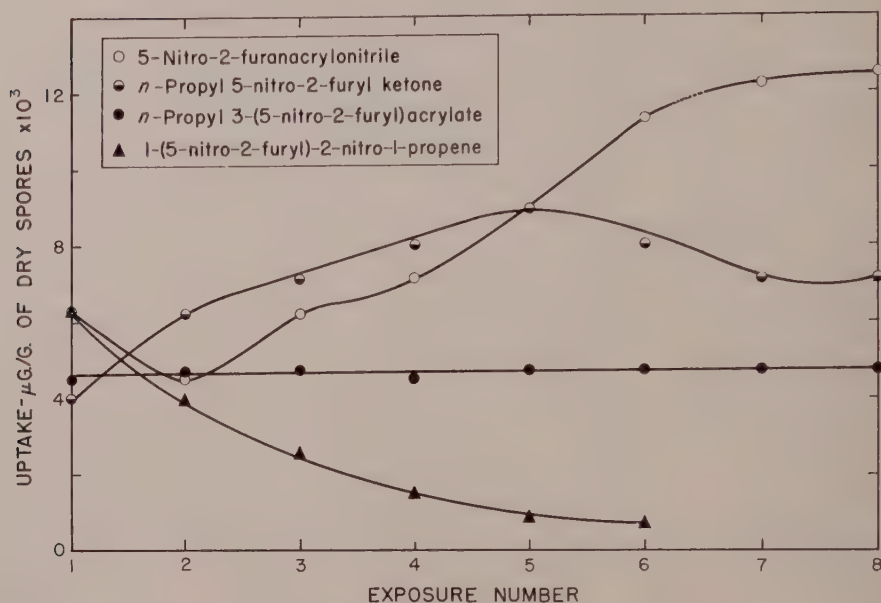


FIGURE 3. Uptake of various 5-nitrofurans by conidia of *Neurospora sitophila* upon repeated exposure of the spores to fresh chemical solution.

were removed from solution after 10 to 15 minutes than after 3 or 4 minutes. Such an uptake pattern can be explained only in terms of release of a portion of the toxicant taken up initially, as suggested earlier by Burchfield and Storrs (2), who observed similar release of *s*-triazine derivatives from *N. sitophila* conidia. A number of the rate curves given in Figure 1 show the tendency toward inversion after various lengths of time, although in several instances the quantities of chemical absorbed in the first few minutes were too small to produce distinct flex points.

#### INHERENT TOXICITY VS. STRUCTURE

Relatively large amounts of most of the compounds were taken up (Table I). However, only a few were found to be fungicidal under the experimental conditions employed. Several of the toxicants were subsequently tested in the conventional slide-germination tests and were found to inhibit germination at dosages well below those to which the spores were exposed in uptake experiments. This suggests that most of the compounds are fungistatic rather than fungicidal at low dosages.

However, three of the compounds in Group II, Table I, were fungicidal, e.g., 1-(5-nitro-2-furyl)-2-nitroethylene, 1-(5-nitro-2-furyl)-2-nitro-1-propene and 3-(5-nitro-2-furyl)acrolein. The ED<sub>50</sub> of each of these in terms of the amount absorbed per gram of oven-dried spores and the correspond-

TABLE II

ED<sub>50</sub> OF SEVERAL 5-NITROFURAN DERIVATIVES FOR *NEUROSPORA SITOPHILA* CONIDIA DETERMINED (A) ON THE BASIS OF ACTUAL AMOUNTS ABSORBED PER GRAM OF SPORES AND (B) BY CONVENTIONAL SLIDE-GERMINATION TEST

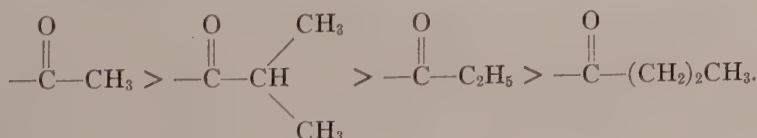
| Compound                                | Approximate ED <sub>50</sub> , p.p.m. |      |
|-----------------------------------------|---------------------------------------|------|
|                                         | (A)                                   | (B)  |
| 3-(5-Nitro-2-furyl)acrolein             | 24,000                                | 2.3  |
| 1-(5-Nitro-2-furyl)-2-nitroethylene     | 8,000                                 | 5.0  |
| 1-(5-Nitro-2-furyl)-2-nitro-1-propene   | 13,000                                | 4.1  |
| Methyl 5-nitro-2-furyl ketone           | 9,000                                 | 1.3  |
| Ethyl 5-nitro-2-furyl ketone            | 20,000                                | 2.5  |
| <i>n</i> -Propyl 5-nitro-2-furyl ketone | 40,000                                | 12.0 |
| Isopropyl 5-nitro-2-furyl ketone        | 15,000                                | 6.0  |

ing ED<sub>50</sub> determined in the conventional slide-germination test are given in Table II.

The data show that a terminal nitro group in the 2-chain rendered the compound highly toxic and was conducive to absorption. Addition of a methyl group reduced both inherent toxicity and absorption. A terminal aldehyde group further reduced toxicity but was about equivalent to the nitro group with regard to absorption. Although 1-(5-nitro-2-furyl)-2-nitro-1-propene was inherently more toxic than the acrolein derivative, each induced about 50 per cent reduction in germination during the third exposure to the chemical. Thus the lower toxicity of the aldehyde was compensated for by greater absorption, so that from the standpoint of practical control of spore germination, the two compounds are about equivalent.

The four compounds in the ketone series (Table II) also reduced germination in the absorption tests. Uptake and inherent toxicity cannot be compared quantitatively with those for the compounds in Section I, Table II, however, because the former were tested at twice the initial concentration of the latter. Preliminary data, as yet unpublished, seem to indicate that the initial concentration of some toxicants has a marked influence on the ED<sub>50</sub>; that is, the higher the initial concentration, the lower the actual quantity of toxicant required for 50 per cent kill on a spore weight basis. The reasons for this are not yet clear.

ED<sub>50</sub> values for the ketones are given in Table II. The descending order of inherent toxicity in terms of the 2-substituent was found to be:





Although the actual amounts of the various derivatives absorbed are different, germination was about equal after the fifth exposure to each chemical. This indicates that differences in inherent toxicity were counter-balanced by differences in absorption; that is, groups which tended to reduce inherent toxicity tended to increase absorption enough to affect germination after about the same number of exposures.

#### DISCUSSION

The present studies show that very large amounts of many of the 5-nitrofurans are absorbed by spores of *N. sitophila*, often without apparent effects on germination. This is in accord with the earlier findings of Miller *et al.* (9) and Burchfield and Storrs (2) who worked with other types of compounds. Their investigations show that toxicants are often absorbed even after viability of the spores has been destroyed. This was found to be true also for some of the nitrofurans, and it suggests that partition of chemicals between the external solution and the spores is not dependent upon metabolic processes concerned with germination. If this is true, the partition must be explained in terms of spore contents. These may be divided into two principal groups, a) lipophilic constituents, i.e., lipids, which make up 15 to 20 per cent of the spore solids (13), and b) hydrophilic material, such as carbohydrates, proteins and nucleic acids, which account for about 70 to 75 per cent of the spore solids (13). It was shown previously that the spore walls, comprising 25 to 30 per cent of the spore weight, have very little, if any, affinity for 2-heptadecyl-2-imidazoline (glyodin), 2,3-dichloro-1,4-naphthoquinone (dichlone) or metal ions (12), and there seems to be no chemical basis to suggest any greater affinity between the wall and nitrofurans. Thus, uptake probably is due to the affinity of the compounds for lipids, proteins and nucleic acids.

Since Overton (7, p. 231) first postulated lipid solubility as the regulatory factor in cellular absorption many workers have attempted to correlate absorption with oil/water partition coefficients. In many instances the correlation has been good. In others it has failed and has given rise to more elaborate theories (7, p. 232). Absorption of the nitrofurans tends to coincide in a general way with expected lipid solubility. However, uptake of certain derivatives cannot be explained on this basis. For example, 1-(5-nitro-2-furyl)-2-nitro-1-propene was absorbed in much smaller quantities than the corresponding aldehyde or nitrile. In general, the more extensive the substitution with hydrocarbon radicals the more lipid-soluble the compound becomes within the molecular-weight limits of the nitrofurans. Thus, on this basis, the propene derivative should be absorbed at least as readily as the corresponding aldehyde or nitrile. Similarly, the isopropyl and *n*-butyl esters in the acrylate series (Group III, Table I) should be at least as lipid-soluble as the aldehyde or nitrile in Group III, but uptake

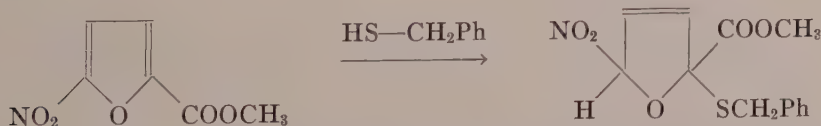
was little more than half that of the latter compounds. It must be concluded, therefore, that oil solubility alone does not regulate absorption.

This conclusion is further necessitated by the fact that in both the acrylate (Group III) and the ketone (Group V) series, the isopropyl derivatives were absorbed in significantly smaller quantities than comparable *n*-propyl derivatives. This suggests that atomic arrangement sometimes has as much influence as the number of atoms.

As was pointed out in a previous section above, the patterns of absorption with regard to time and upon repeated exposure suggest that some of the compounds induced an increase in permeability of the spore membrane and perhaps that of intracellular particles such as mitochondria and microsomes. At present, however, no evidence is available on the nature of membrane constituents that might be involved.

The data on inherent toxicity vs. absorption serve to point out which substituent groups contribute most to toxicity and which tend to induce greater absorption. Although much has been learned from screening programs in which only the external concentration of chemical is known, a catalogue of comparisons of different groups on various nuclei between relative effects on uptake and inherent toxicity would undoubtedly provide new ideas to the chemist concerned with synthesis of new biocides. Unfortunately, many of the present compounds were not fungicidal at the dosages received by the spores under the condition of the present experiments. However, information on structure vs. absorption without toxicity data can be useful in synthesis of new derivatives containing known thiophores.

The data on toxicity show that the 2-substituent had a strong influence on inherent toxicity as well as on absorption. It is apparent, therefore, that the reactivity of the various compounds was influenced in an important way by the 2-substituent. Dann and Möller (3, 4) have pointed out that the chemical action might be due to the easily reducible 5-nitro group. Recent findings of Irie and Kurosawa (8) suggest that the effects of the 2-substituent might be through inductance, because they were able to demonstrate the addition of a thiol and sodium methoxide to methyl 5-nitrofuroate:



That either or both of these kinds of interactions can occur in biological systems seems to be supported by a considerable volume of data showing interference of 5-nitrofurans with various enzymes in bacteria and in animal tissues (5, 6, 14, 15). Current studies on the effects of several deriva-

tives on organic acid metabolism in fungus spores also indicate interference with citrate synthesis and pyruvate metabolism.

Comparison of uptake and toxicity data with the data on disease control (11) shows a degree of general correlation. For example, compounds containing an ethylenic double bond in the 2-substituents were the most effective of the series against early and late blights of tomato and powdery mildew of bean. Of all compounds tested 1-(5-nitro-2-furyl)-2-nitroethylene, 1-(5-nitro-2-furyl)-2-nitro-1-propene and 3-(5-nitro-2-furyl)acrolein were most effective. These same compounds were found to be taken up readily by spores and to be inherently the most toxic.

A number of the compounds were quite active in the foliage tests but were not lethal in the uptake tests. They were taken up in considerable quantities on long exposure and are probably fungistatic at moderate dosages, which would account for the observed disease control.

The data indicate that even with knowledge of uptake and inherent toxicity, it is not possible to predict foliage performance with adequate precision. On the other hand, sufficient knowledge of the influence of substituents on uptake, inherent toxicity, stability and other factors would undoubtedly serve as a valuable general guide in synthesis of new biocides.

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# Note

## GROUP INTERACTIONS IN THE REACTIONS OF PIPERAZINE AND PIPERIDINE WITH 1-FLUORO-2,4-DINITROBENZENE

H. P. BURCHFIELD AND ELEANOR E. STORRS

Previous investigations on the reactivities of amino acids and peptides with 1-fluoro-2,4-dinitrobenzene (FDNB) and *s*-triazine derivatives in aqueous buffers showed that only the ionized amino and sulfhydryl groups are able to react at appreciable rates; the  $\text{RNH}_3^+$  and RSH groups being either inert or extremely sluggish (1, 2). Consequently, intramolecular group interactions which decrease the pK values of functional groups tend to increase apparent chemical reactivities in solutions buffered at pH values near neutrality. However, the same factors which contribute to pK lowering often reduce nucleophilicity so that the increase in apparent reactivity is usually much less than would be predicted from the change in degree of ionization alone.

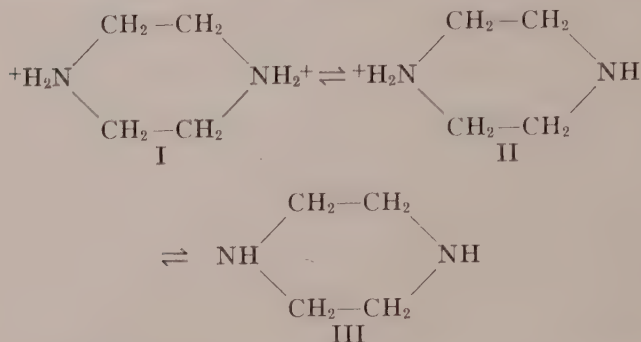
Sometimes the various opposing effects cancel each other altogether. Thus, cysteine and glutathione react with FDNB at the same apparent rates at pH 7, even though the sulfhydryl group of the former compound is much more highly ionized (2). To study this effect further with molecules having approximately the same size and shape, the reactivities of piperazine and piperidine with FDNB were determined by methods described previously (1, 2). Piperidine contains a single secondary amino group with a pK value of 11.12. Piperazine contains two secondary amino groups with pK values of 5.56 and 9.80. Consequently aqueous solutions of piperazine buffered at pH 7 should contain about  $10^4$  times more uncharged NH groups than equimolar solutions of piperidine. This should tend to promote reactivity.

Experimental evidence confirms that the apparent reactivity of piperazine in *M*/30 phosphate buffer at pH 7 is about 30 times higher than that of piperidine on a molar basis, or about 15 times higher on an equivalent (NH) basis (Table I). However, this difference is considerably less than would be predicted from degree of ionization alone.

TABLE I  
APPARENT REACTIVITIES OF PIPERAZINE AND PIPERIDINE WITH  $2.7 \times 10^{-5}$   
MOLAR SOLUTIONS OF 1-FLUORO-2,4-DINITROBENZENE

| Reactant   | pH  | Molar concn.<br>of amine | Apparent<br>velocity coefficient<br>(moles liter <sup>-1</sup> sec. <sup>-1</sup> ) |
|------------|-----|--------------------------|-------------------------------------------------------------------------------------|
| Piperidine | 7.0 | 0.081                    | 0.00102                                                                             |
| Piperidine | 8.0 | 0.0108                   | 0.0108                                                                              |
| Piperazine | 7.0 | 0.0027                   | 0.0310                                                                              |
| Piperazine | 8.0 | 0.000675                 | 0.145                                                                               |

The ionization of piperazine can be described formally as follows:



The doubly charged cation (I) has little if any reactivity and its concentration in aqueous solution at pH 7 is low. Consequently, its contribution to reactivity can be neglected. The singly charged cation (II) can be depicted as a mixture of identical tautomers, or perhaps more accurately as a piperazine ring associated with a mobile proton. For convenience in calculation it is considered here as an uncharged reactive group coupled with a charged deactivating group. The neutral molecule (III) contains two reactive NH groups which tend to deactivate each other but with considerably less intensity than the  $\text{NH}_2^+$  group of II. On increasing the pH of aqueous solutions of piperazine from 7 to 8, the concentration of II will increase by less than 2 per cent so its contribution to total reactivity will not change appreciably. However, the concentration of III, and consequently its contribution to reactivity, will increase about 10-fold. A 4.7-fold increase in apparent chemical reactivity was actually obtained on changing pH from 7 to 8, indicating participation by both ionic species. Calculations from these data show that 60 per cent of the reactivity of piperazine at pH 7 is contributed by II, and the remaining 40 per cent by III. At pH values of 8 and above, III is the predominant ionic species contributing to reactivity.

The velocity coefficient for the reaction of II with FDNB is about  $2 \times 10^{-2}$  mole liter<sup>-1</sup> sec.<sup>-1</sup> at 29° C. assuming one reactive imino group per molecule. The velocity coefficient for the reaction of III with FDNB is 8 mole liter<sup>-1</sup> sec.<sup>-1</sup>, but there are two reactive imino groups present in each molecule. Thus, the reactivity of each imino group of III is about 200 times higher than the corresponding group of II although II makes the greater contribution to reactivity at pH 7 because of its lower pK value.

The apparent reactivity of piperidine at pH 7 is only about 3 per cent of the value found for piperazine on a molar basis. However, when degree of ionization is taken into account, the intrinsic velocity coefficient for the reaction of the uncharged molecule with FDNB is found to be 13 mole liter<sup>-1</sup>

sec.<sup>-1</sup> at 29° C. Thus, the reactivity of the imino group of piperidine is approximately 3 times higher than the corresponding group of III, and about 650 times higher than the corresponding group of II. Evidently the NH groups of piperazine are sufficiently electronegative to deactivate each other. When one or the other of the groups becomes protonated, reduction in reactivity is much more severe because of the strong electron-attracting capacity of the NH<sub>2</sub><sup>+</sup> group. However, the apparent reactivity of piperazine in solutions buffered at pH 7 is considerably greater than that of piperidine since the reduction in nucleophilicity is more than counterbalanced by a very large increase in degree of ionization.

This suggests that some groups with high latent nucleophilicity may react sluggishly at pH values near neutrality because of their failure to ionize. Thus, the hydroxyls of serine and threonine do not react with FDNB and similar reagents at pH 7, even though the ionized aliphatic hydroxyl group is potentially one of the most nucleophilic centers possible in biological systems. By contrast, the sulfhydryl group has a widespread reputation for high reactivity, which is probably based on its capacity to ionize to a much greater extent than the hydroxyl group at pH 7, as well as on its high innate nucleophilicity.

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# MECHANISM OF ACTION OF THE FUNGICIDE CAPTAN [*N*-(TRICHLOROMETHYLTHIO)-4-CYCLOHEXENE-1,2-DICARBOXIMIDE]<sup>1</sup>

ROBERT G. OWENS AND HELEN M. NOVOTNY

## SUMMARY

Endogenous respiration of spores of *Neurospora sitophila* was inhibited 80 to 90 per cent by lethal doses of captan. CO<sub>2</sub> formation was inhibited somewhat less than O<sub>2</sub> uptake. As a result, the R.Q. increased progressively with increasing dosages of toxicant. Addition of glucose, acetate, glycerol or ethanol to the incubation medium failed to increase O<sub>2</sub> consumption by poisoned spores.

Endogenous metabolism tended to recover from the effects of small amounts of captan after an hour, indicating reversal of inhibition. Viability of the spores showed the same tendency to recover. The probable basis of recovery is discussed in terms of decomposition of the toxicant. Toxicity is correlated with the amount of captan absorbed by the spores, and an excess of captan in the ambient solution is necessary to kill the spores. When all available captan is absorbed, metabolism and viability of the spores tend to recover. The biological evidence is inconsistent with the idea that decomposition products of captan, such as thiophosgene, are the primary toxicants in captan action.

Lipids and protein decreased in poisoned spores in the same manner as in normal spores upon aerobic incubation for 16 hours. CO<sub>2</sub> formation and carbohydrate and amino acid dissimilation were somewhat inhibited by lethal doses of captan.

Inorganic phosphate accumulated in poisoned spores, regardless of whether various phosphate acceptors were added. This indicates that phosphorus metabolism was blocked. Nucleic acid and lipid phosphorus decreased.

Pyruvate,  $\alpha$ -ketoglutarate and two unidentified keto acids accumulated to very high levels in accordance with inhibition of germination. Citrate synthesis from acetate was also inhibited in proportion to inhibition of germination, apparently because of inactivation of coenzyme A.

It is concluded that the toxicity of captan is due to reactions involving the intact captan molecule, not its decomposition products. Key factors in its toxic action appear to be inhibition of a number of enzymes in phosphorus metabolism, certain oxidases and dehydrogenases, carboxylases and coenzyme A. A number of reactions were found to proceed in poisoned spores, but they are almost entirely catabolic and cannot support growth.

## INTRODUCTION

Since its introduction in 1952 (4), captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] has become an important commercial fungicide, and a number of studies have been made toward elucidation of its mode of action. Hochstein and Cox (3) found that captan inhibits yeast carboxylase *in vitro*. Silber (9) showed that other compounds contain-

<sup>1</sup> This work was supported in part by a grant from the Rockefeller Foundation. Copyright, 1959, by Boyce Thompson Institute for Plant Research, Inc.

ing the *N*-(trichloromethylmercapto) group also inhibit carboxylase, the relative potency of different derivatives being dependent upon the nature of other substituent groups. Duggar *et al.* (2) reported that yeast hexokinase is inactivated by captan, which suggests that toxicity might be the result of inactivation of enzymes other than, or in addition to, carboxylase. This is further suggested by the fact that Hochstein and Cox were able to reverse captan-induced inhibition of carboxylase *in vitro* by adding cocarboxylase to the system, but were unable to reverse inhibition of the growth of cells by addition of thiamine pyrophosphate or thiamine.

The present experiments were carried out to determine the relative importance of inhibition of various enzymes and metabolic processes in the toxic action of captan. Effects of the fungicide on a number of the major phases of intermediary metabolism have been determined and related to effects on spore germination.

#### MATERIALS AND METHODS<sup>2</sup>

Captan was purified from a commercial sample of 50 per cent wettable powder. Crystals melting at 171–172° C. were used for all experiments.

Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge were used as the test subject. Methods of culture, harvesting, determination of dry weight and preparation of the conidia for the tests were described previously (8).

Respirometry was conducted with the Warburg apparatus (10) at 29° C. Each vessel contained approximately 10 mg. of spores (dry weight), 2 mg. of substrate when appropriate, and various amounts of captan as stated in the tables and figures. The total volume was 2 ml.

Captan was dissolved in acetone, diluted as desired, and added in 0.1 ml. of acetone to the spores and other components of the system in the main compartment of the flask. An identical portion of acetone without captan was added to the controls. The center well contained 0.2 ml. of 10 per cent KOH solution or 0.2 ml. of water, as appropriate, and a 2×2 cm. square of fluted filter paper.

Respiratory quotients were determined by Warburg's direct method (10, p. 28) and are based on O<sub>2</sub> and CO<sub>2</sub> values obtained during the first 30 minutes of the experiments. Q<sub>O<sub>2</sub></sub> and Q<sub>CO<sub>2</sub></sub> values are also based on the first 30 minutes unless otherwise stated.

Methods of extraction and assay of various components and procedures for determining viability of the spores were described or cited previously (7).

Uptake of captan was determined according to the assay method de-

<sup>2</sup> Abbreviations and symbols used in this paper are: Q<sub>O<sub>2</sub></sub>=μl. O<sub>2</sub>/hr.×mg. dry wt. of spores<sup>-1</sup>; Q<sub>CO<sub>2</sub></sub>=μl. CO<sub>2</sub>/hr.×mg. dry wt. of spores<sup>-1</sup>; R.Q.=respiratory quotient.

scribed by Wagner *et al.* (11), except that the reaction was carried out in 75 instead of 100 per cent acetone. This modification was made to eliminate the necessity of extracting the captan from incubation solutions. The spores were incubated on a reciprocating shaker for various periods of time up to 60 minutes. The systems contained approximately 50 mg. (dry weight) of spores and the amounts of captan stated in the tables and figures. The toxicant was added in 0.1 ml. of acetone to 5.9 ml. of aqueous spore suspension.

After the incubation period, a drop of the spore suspension was diluted in 50 ml. of distilled water for determination of viability (7). Nineteen ml. of acetone were added and mixed with the remaining spore suspension. One ml. of this mixture was then added to the reagents (11) in a 15-ml. clinical centrifuge tube. After 20 minutes, when the color was fully developed, the spores were removed by centrifugation and the color density was measured with a Beckman Model DU spectrophotometer. The amounts of captan left in solution were calculated by reference to appropriate standards. Development of the color was unaffected by the presence of spores.

## RESULTS

### EFFECTS OF CAPTAN ON ENDOGENOUS METABOLISM

The effects of captan at several doses on respiration and germination are shown in Figures 1 and 2. Sublethal doses of the fungicide induced slight acceleration of  $O_2$  uptake and  $CO_2$  release. As the concentration was increased, respiration was inhibited proportionately up to 80 to 90 per cent. Consumption of  $O_2$  amounting to 10 to 20 per cent of the original continued even at hyperlethal<sup>3</sup> doses. Carbon dioxide formation was inhibited somewhat less than  $O_2$  uptake, as shown by comparison of data in parts A and B, Figure 2. As a result, the respiratory quotient (R.Q.) increased progressively with increasing dosages of toxicant (Fig. 1 C).

The effects of captan on germination, on oxygen consumption and on the R.Q. of the spores are compared with regard to captan concentration in Figures 1 and 2 C. It can be seen that more captan was required to inhibit germination than to inhibit endogenous metabolism or to induce the rise in R.Q. in short-term experiments. This suggests that endogenous metabolism was more sensitive than germination to the toxicant. However, simple logic holds that growth cannot occur without metabolism. Thus, the cause of the discrepancy was sought in terms of differences in time between the relatively short-term respiratory experiments and the time required for the spores to germinate. This is discussed in a subsequent section.

<sup>3</sup> "Hyperlethal" is used in the medical sense and refers to doses in excess of the amount required to inhibit germination 100 per cent.



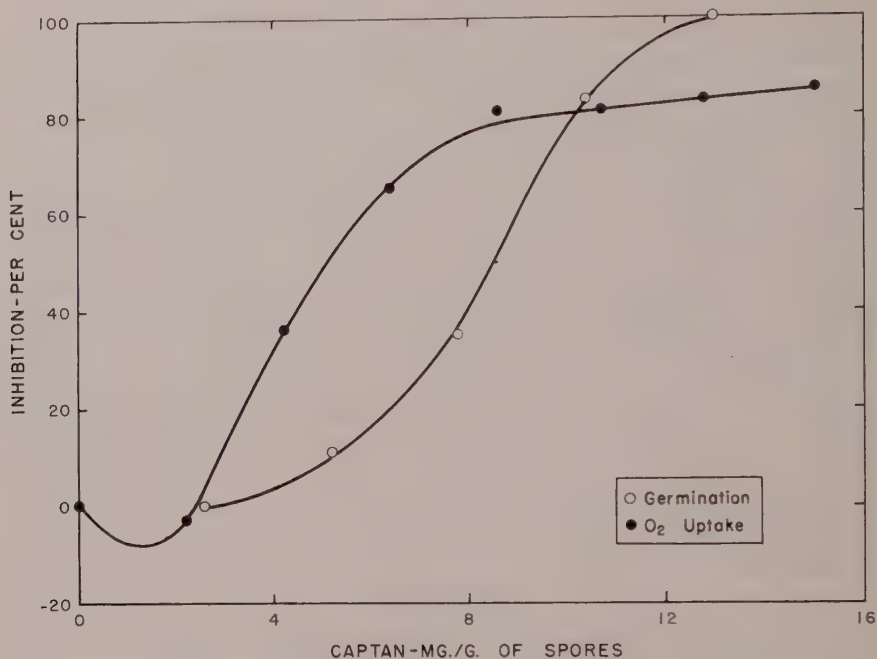


FIGURE 1. Effect of captan at various concentrations on oxygen uptake and germination of conidia of *Neurospora sitophila*.

#### EFFECTS OF CAPTAN ON RESPIRATION OF VARIOUS METABOLIC INTERMEDIATES

The effects of captan on endogenous respiration have already been shown by the data in Figure 2. Oxidation of various intermediates normally metabolized by the spores was also inhibited (Table I). Glucose and acetate, which induced large increases in  $Q_{O_2}$  and  $Q_{CO_2}$  in untreated spores, were not oxidized in the presence of lethal amounts of captan. Oxidation of ethanol and glycerol was also substantially inhibited by the higher dose of captan. However,  $CO_2$  release in the presence of glycerol was particularly persistent, even at the higher dose of captan, and  $O_2$  uptake continued at a somewhat more rapid rate than with other intermediates tested. The persistent release of  $CO_2$ , which was probably due to endogenous substrates rather than those added, was also apparent in carbon-balance studies reported in a subsequent section.

The endogenous R.Q. in these experiments was somewhat higher than found previously (7). This might have been due to the small amount of acetone that was included in the controls in order to make them compara-

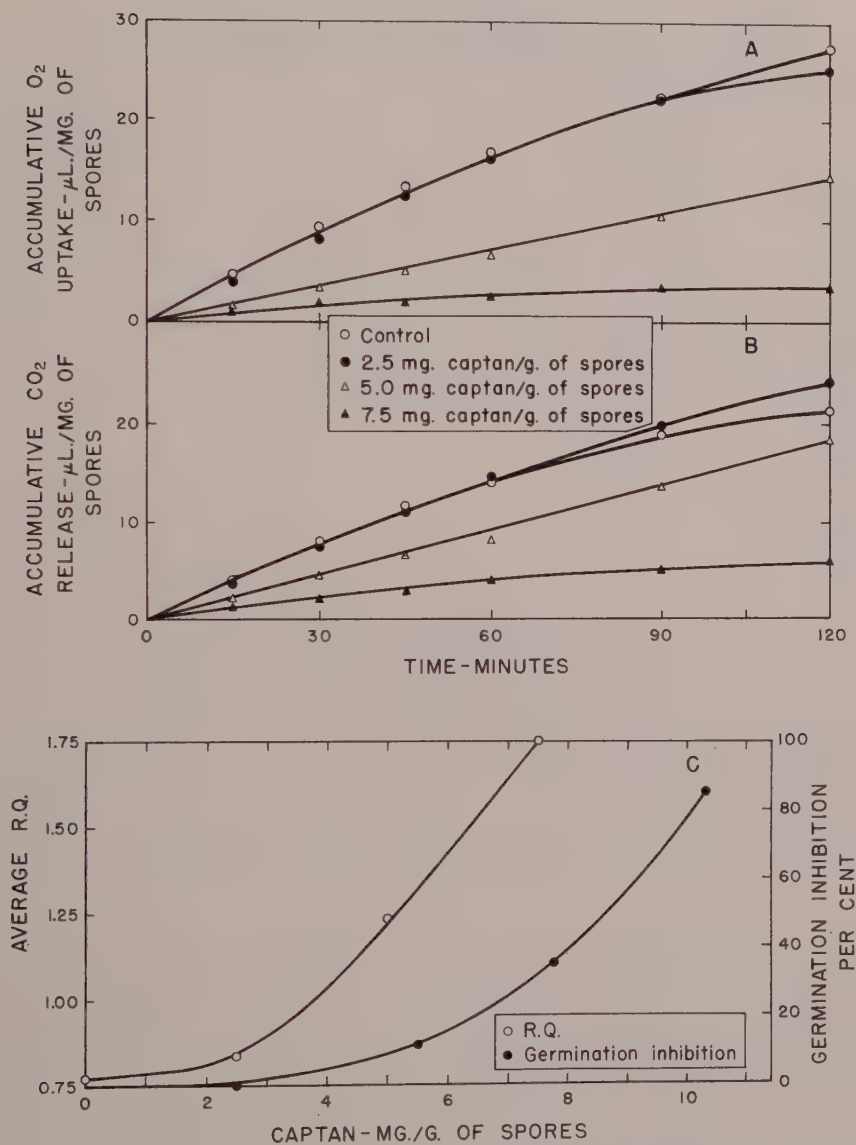


FIGURE 2. Effect of captan at several concentrations on (A) oxygen uptake, (B)  $CO_2$  release and (C) the respiratory quotient of conidia of *Neurospora sitophila* in relation to time.

TABLE I

EFFECTS OF CAPTAN ON RESPIRATION OF CONIDIA OF *NEUROSPORA SITOPHILA*  
INCUBATED WITH VARIOUS METABOLIC INTERMEDIATES

| Metabolite* | Captan<br>concentration,<br>mg./g. of<br>spores** | Q <sub>O<sub>2</sub></sub> | Q <sub>CO<sub>2</sub></sub> | R.Q. | Inhibition, %              |                             |
|-------------|---------------------------------------------------|----------------------------|-----------------------------|------|----------------------------|-----------------------------|
|             |                                                   |                            |                             |      | Q <sub>O<sub>2</sub></sub> | Q <sub>CO<sub>2</sub></sub> |
| None        | None                                              | 17.4                       | 14.4                        | .83  | —                          | —                           |
| Glucose     | None                                              | 24.8                       | 25.6                        | 1.03 | —                          | —                           |
| Glucose     | 7.2                                               | 3.6                        | 6.4                         | 1.78 | 85                         | 75                          |
| Glucose     | 14.3                                              | 0.4                        | 3.0                         | 7.50 | 98                         | 88                          |
| None        | None                                              | 14.5                       | 11.6                        | .80  | —                          | —                           |
| Acetate     | None                                              | 20.0                       | 14.4                        | .72  | —                          | —                           |
| Acetate     | 9.3                                               | 4.4                        | 7.0                         | 1.59 | 78                         | 51                          |
| Acetate     | 18.6                                              | 0.6                        | 2.4                         | 4.00 | 97                         | 83                          |
| None        | None                                              | 7.2                        | 5.6                         | .78  | —                          | —                           |
| Glycerol†   | None                                              | 8.4                        | 6.5                         | .77  | —                          | —                           |
| Glycerol†   | 6.9                                               | 8.2                        | 8.4                         | 1.02 | 2                          | +29                         |
| Glycerol†   | 13.8                                              | 1.8                        | 3.2                         | 1.78 | 79                         | 51                          |
| None        | None                                              | 10.5                       | 8.6                         | .82  | —                          | —                           |
| Ethanol     | None                                              | 17.9                       | 15.7                        | .88  | —                          | —                           |
| Ethanol     | 6.7                                               | 6.2                        | 15.9                        | 2.56 | 65                         | +1                          |
| Ethanol     | 13.3                                              | 0.7                        | 3.7                         | 5.28 | 96                         | 76                          |

\* The concentration of each metabolite was approximately 400 mg./g. of spores.

\*\* Variations in metabolite and captan concentrations relative to the weight of spores are due to differences in dry weight of a unit volume of spores from test to test.

† The Q<sub>O<sub>2</sub></sub> and Q<sub>CO<sub>2</sub></sub> for glycerol are based on the 30-minute period between 90 and 120 minutes. Increases induced by glycerol in the first hour were too small to be decisive. Since the respiration rate decreased in the 90-minute interim the O<sub>2</sub> and CO<sub>2</sub> quotients are somewhat lower than those based on the first 30 minutes.

ble to those samples where captan was added in acetone. The acetone concentration, however, was not sufficient to inhibit germination and does not invalidate comparisons of the various treatments.

## RECOVERY OF METABOLISM FROM THE TOXIC EFFECTS OF CAPTAN

The effects of captan at three concentrations on O<sub>2</sub> consumption and CO<sub>2</sub> release are shown in Figure 2, A and B. At 2.5 mg./g. of spores, captan caused slight acceleration of respiration. Five mg./g. induced moderate retardation and 7.5 mg./g. resulted in about maximum inhibition over a period of 2 hours. At 5 mg./g., however, the curves for O<sub>2</sub> uptake and CO<sub>2</sub> release are slightly concave or linear instead of convex. This suggests that the rate of these functions increased with time, whereas it normally decreased when no toxicant was present. That this is true is shown in Figure 3 where the Q<sub>O<sub>2</sub></sub> and Q<sub>CO<sub>2</sub></sub> at various times up to two hours are compared with those of untreated spores. The Q<sub>O<sub>2</sub></sub> and Q<sub>CO<sub>2</sub></sub> of untreated spores fell off to less than half of the maximum. The Q<sub>O<sub>2</sub></sub> of treated spores gradually increased, after an initial period of strong inhibition, until it was slightly

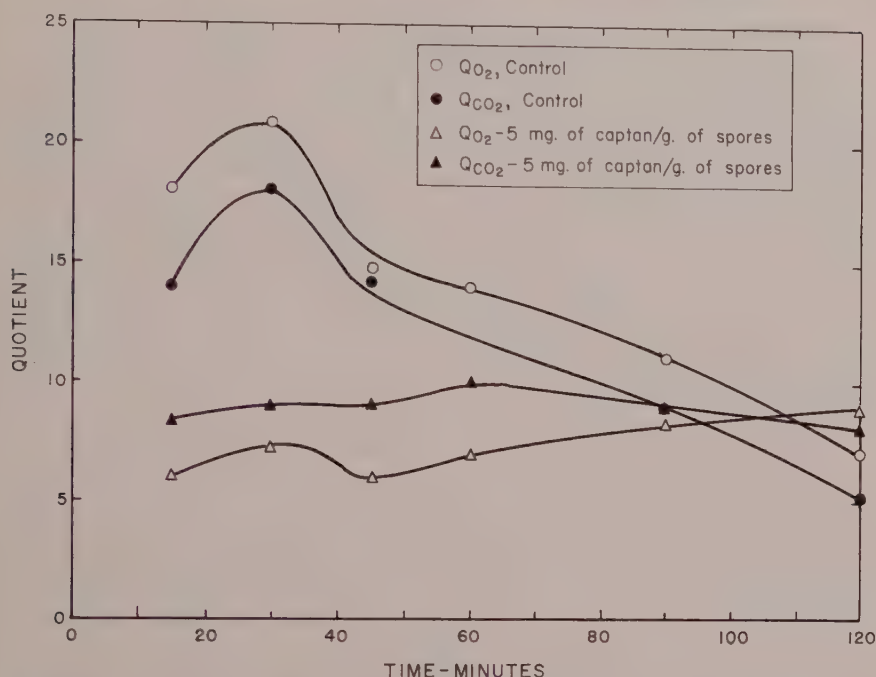


FIGURE 3. Recovery of conidia of *Neurospora sitophila* with time at an initial dose of 5.0 mg. of captan/g. of spores.

higher than that of the control after two hours. The  $Q_{CO_2}$  remained essentially constant after the first hour but was considerably above that of the controls after two hours due to the decrease in  $CO_2$  release in the controls. The data indicate, therefore, that endogenous metabolism was strongly affected by sublethal amounts of captan initially, but that the effects gradually wore off.

Recovery of metabolic functions implies that the inhibitory interaction of captan with certain enzymes is reversible at sublethal doses. Reversibility further implies a decrease in captan concentration or an increase in enzyme concentration. There is no evidence to support the latter, but the former is supported by the findings of Burchfield and Schechtman (1) and Lukens and Sisler (5), who showed that captan is unstable in aqueous solution. The half-life at pH 7.0 in buffered solution was found to be about 2.5 hours (1). The present data show that basal metabolism recovered to a considerable extent from the effects of 5 mg. of captan/g. of spores in two hours, when 40 to 45 per cent, or about 2.0 to 2.2 mg., of the original captan would be expected to decompose. The data in Figure 1 show that the remainder, 3.0 mg. of captan, would have little effect on  $O_2$  uptake.

Reversal of inhibition as a result of captan decomposition makes the



inconsistency between inhibition of endogenous metabolism and germination more intelligible. Determination of spore germination requires eight hours or more at room temperature. During this time more than 90 per cent of the initial captan can be expected to decompose, even if the rate of decomposition is not accelerated by the spore contents. Thus, unless the spores were killed almost instantaneously, which is counter-indicated by recovery of metabolic functions at low dosages, or unless breakdown products were responsible for toxicity, as suggested by Lukens and Sisler (5), decomposition would be an important factor in comparing long- and short-term experiments. This would account for the difference in concentration required to inhibit respiration, a short-term determination, and germination, a relatively long-term determination.

#### ABSORPTION AND DECOMPOSITION OF CAPTAN IN RELATION TO GERMINATION OF THE SPORES

Recently it has become increasingly evident that knowledge of the actual dose of toxicant absorbed by fungus spores is essential for an understanding of the efficiency of fungicides (6). This would appear to be especially true for toxicants such as captan, elemental sulfur and certain other sulfur-containing fungicides, which undergo rather rapid changes in solution and in living cells. To explore the possible relationship between decomposition products of captan and toxicity (5), a study was made in which viability, uptake and breakdown of captan were compared in relation to time. Figure 4, A-C, shows the rate of disappearance of captan at three initial concentrations when exposed to spores of *N. sitophila* in aqueous solution and the corresponding effects on germination. The curves represent a composite measure of absorption by the spores and decomposition, since only the toxicant remaining in solution could be measured.

Disappearance of captan was extremely rapid, which is in accord with earlier findings for uptake of other toxicants (6). The ED<sub>50</sub> values expressed on a dry spore weight basis are given in Table II. The ED<sub>50</sub> depended upon when the germination counts were made, i.e., whether they were made immediately after the controls were fully germinated or later. However, the amount of captan required to inhibit germination of 50 per cent of the spores was essentially the same at any given time whether the initial dose was 6.3, 8.5 or 10.3 mg./g. of spores. The higher ED<sub>50</sub> obtained in the later counts shows that germination of many spores was only retarded and that the effects of the toxicant wore off in time. This is in accord with the data on respiration in the previous section.

Although absorption and decomposition could not be assayed separately in the presence of spores, approximate decomposition values based on the decomposition rates in buffered solutions (1) are included in Figure 4, A-C. It is likely that decomposition within the cytoplasm would proceed

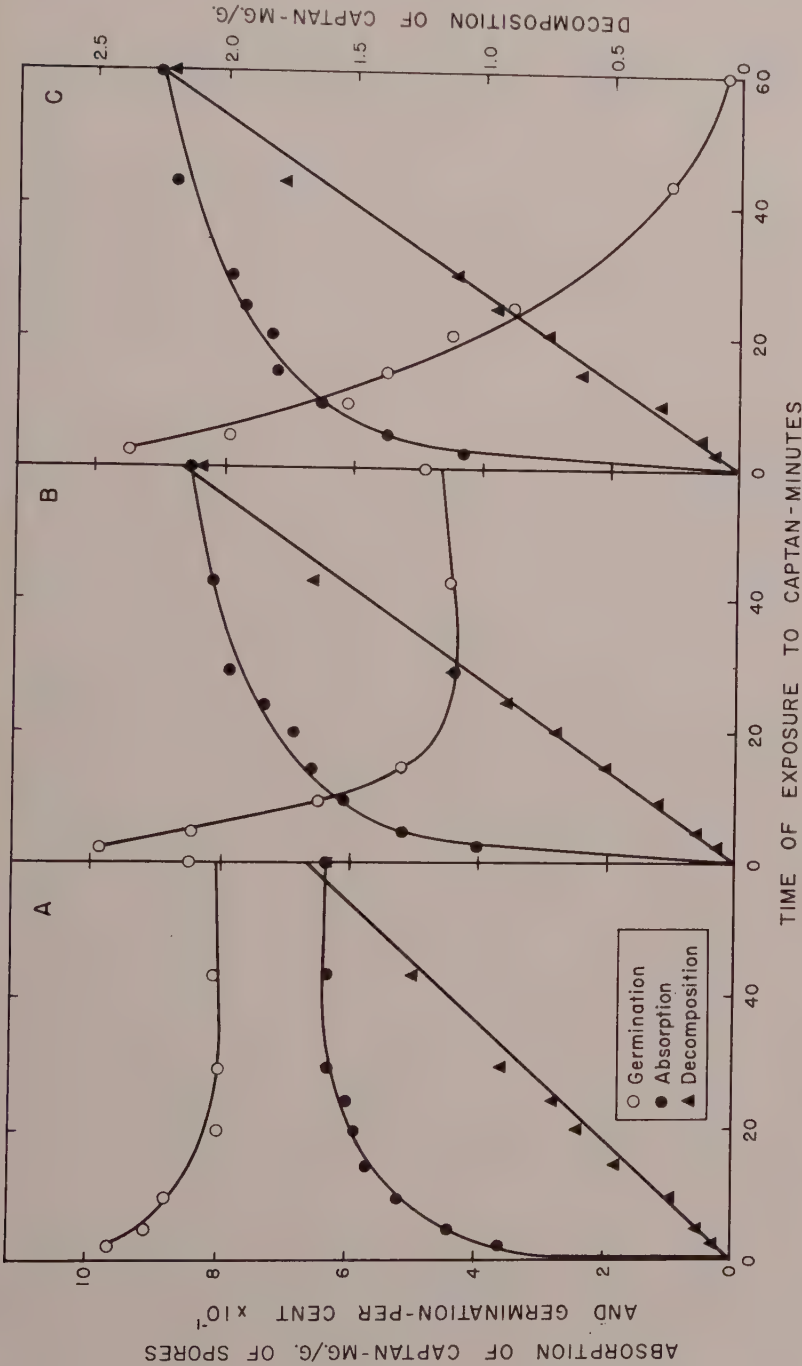


FIGURE 4. Uptake, decomposition and germination of conidia of *Neurospora sitophila* in relation to time at initial doses of (A) 6.3, (B) 8.5 and (C) 10.3 mg. of captan/g. of spores.

TABLE II

ED<sub>50</sub> OF CAPTAN FOR CONIDIA OF *NEUROSPORA SITOPHILA* ON THE BASIS OF THE AMOUNT ABSORBED.\* COUNTS MADE 8 AND 12 HOURS AFTER PLACING SPORES IN GERMINATION MEDIUM MAINTAINED AT ROOM TEMPERATURE

| Initial dose,<br>mg./g. of spores | ED <sub>50</sub> —mg./g. of spores |               |
|-----------------------------------|------------------------------------|---------------|
|                                   | 8-Hour count                       | 12-Hour count |
| 6.3                               | 6.0                                | —             |
| 8.5                               | 5.2                                | 7.5           |
| 10.3                              | 5.6                                | 6.8           |
| 12.4                              | 5.4                                | 6.6           |

\* Oven-dry weight of spores.

somewhat more rapidly than in buffered solution, since Burchfield<sup>4</sup> has obtained evidence that breakdown is ion-catalyzed. The important point here, however, is not the absolute rate of breakdown, but the fact that breakdown, based on the amount of captan absorbed, proceeds in a linear manner over a period of 60 minutes when only about 25 per cent of the amount absorbed should be decomposed. This would leave 75 per cent of that absorbed to continue producing breakdown products. The germination data, however, for initial doses of 6.3 and 8.5 mg. of captan/g. of spores show that inhibition was maximal but not complete after about 20 minutes even though decomposition products continued to be formed at the same rate for much longer periods of time. It seems unlikely, therefore, that inhibition of germination is dependent upon the formation of decomposition products as suggested by Lukens and Sisler (5). This is further indicated by the data in Figure 5 which show that inhibition of germination was directly and more or less linearly related to the amount of captan absorbed. At initial doses of 6.3 and 8.5 mg. of captan/g. of spores, inhibition ceased when all of the captan was taken up although decomposition of the toxicant would continue within the spores at the same rate as before. The later counts show that despite the continued formation of breakdown products, many of the spores recovered the ability to germinate when no excess captan was present. It must be concluded, therefore, that toxicity of captan is due to the intact captan molecule, not to decomposition products, and that an excess of intact captan must be available to effect complete and permanent inhibition of germination.

#### EFFECTS OF CAPTAN ON METABOLISM OF SOME MAJOR SPORE COMPONENTS

It was shown in previous sections dealing with respiration that release of CO<sub>2</sub> continued to a considerable extent in poisoned spores. The origin

<sup>4</sup> Personal communication.

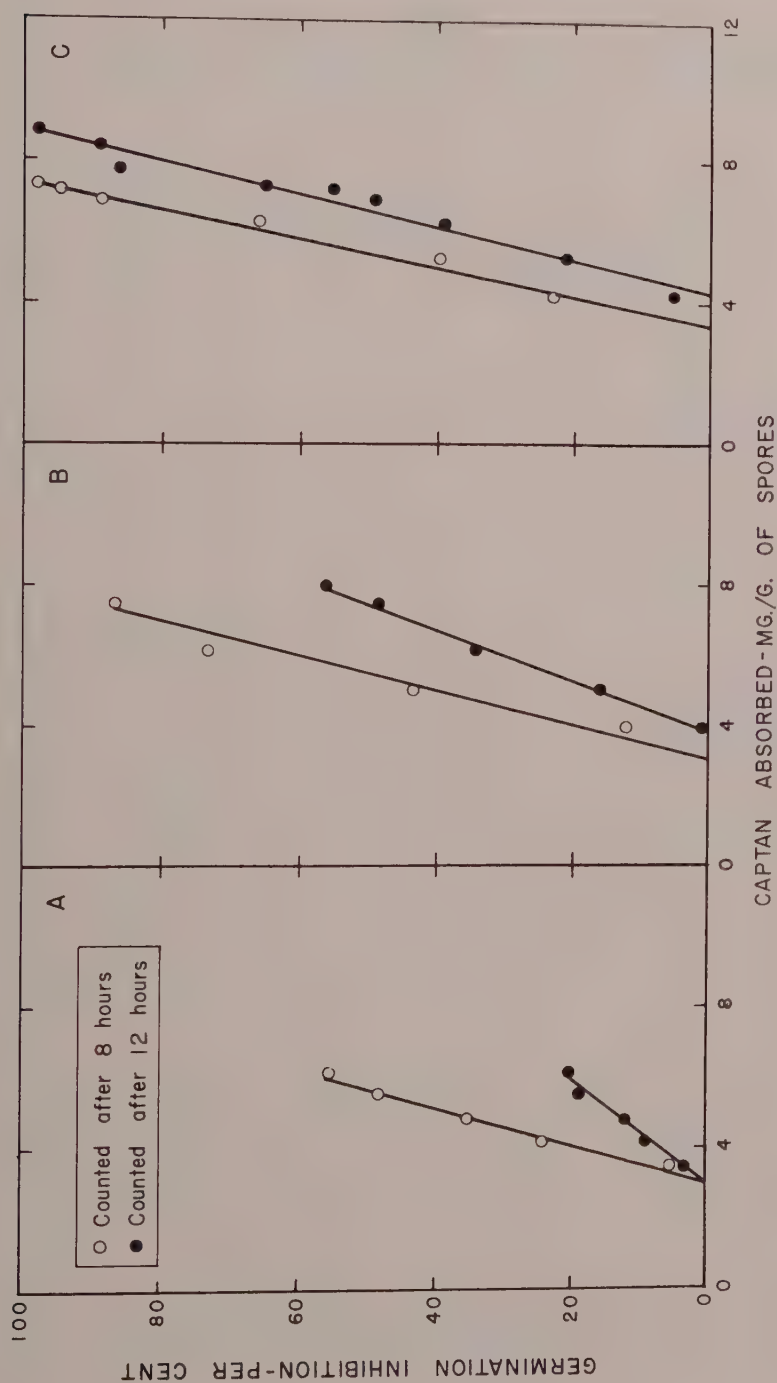


FIGURE 5. Germination of conidia of *Neurospora sitophila* in relation to the amount of captan absorbed (mg./g. of oven-dried spores) at initial doses of (A) 6.3, (B) 8.5 and (C) 10.3 mg. of captan/g. of spores.



TABLE III

EFFECT OF CAPTAN AT THE LETHAL DOSAGE ON THE AMOUNTS OF CO<sub>2</sub> RELEASED, AND ON CARBOHYDRATE, LIPID, PROTEIN AND OTHER COMPONENTS OF *NEUROSPORA SITOPHILA CONIDIA* INCUBATED AEROBICALLY WITH AND WITHOUT ACETATE AS A SUBSTRATE

| Treatment          | Substrate | CO <sub>2</sub> released, mg./g. of spores | Spore constituents, mg./g. |       |         |         | Total spore constituents assayed + CO <sub>2</sub> , mg./g. | Change from incubated control, % |
|--------------------|-----------|--------------------------------------------|----------------------------|-------|---------|---------|-------------------------------------------------------------|----------------------------------|
|                    |           |                                            | Carbohydrate               | Lipid | Protein | Residue |                                                             |                                  |
| Before incubation* | None      | None                                       | 93                         | 177   | 258     | —       | —                                                           | —                                |
| Incubated          | None      | 111                                        | 114                        | 46    | 113     | 320     | 704                                                         | —                                |
| Incubated/captan** | None      | 46                                         | 59                         | 45    | 108     | 316     | 574                                                         | -19                              |
| Incubated          | Acetate†  | 204                                        | 120                        | 41    | 74      | 418     | 857                                                         | +21                              |
| Incubated/captan** | Acetate†  | 41                                         | 53                         | 44    | 76      | 347     | 561                                                         | -20                              |

\* Data from Owens, Novotny and Michels (8).

\*\* 21.2 Mg. of captan/g. of spores.

† Approximately 400 mg. of acetate/g. of spores.

of the CO<sub>2</sub> was determined in a study of carbon balance between major components of the spores and CO<sub>2</sub> in poisoned and in untreated spores. It was found that lipids and protein decreased to less than half the original values upon incubating the spores aerobically for 16 hours (Table III). Some, but not all, of the decrease was accounted for by the CO<sub>2</sub> formed. Hot-water-soluble carbohydrates increased somewhat, possibly as a result of hydrolysis of glycoprotein with release of the sugar moiety and breakdown of lipids with release of glycerol, which reacts like carbohydrate in the analytical procedure.

Acetate almost doubled CO<sub>2</sub> release from untreated spores and induced a substantial increase in the residue fraction, which consists largely of spore wall material. It had little effect on lipid dissimilation but apparently accelerated protein breakdown and, to some extent, increased water-soluble carbohydrates.

Although treatment of the spores with a lethal dose of captan resulted in more than 50 per cent reduction in CO<sub>2</sub> formation, an important volume of CO<sub>2</sub> continued to be formed (Table III). This agrees with the experiments on respiration reported above. Acetate had no effect on CO<sub>2</sub> formation in poisoned spores, which is also in accord with findings in studies on respiration. Breakdown of protein and lipids proceeded as in untreated spores.

The principal difference between treated and untreated spores occurred in the hot-water-soluble carbohydrates, which decreased about 50 per cent in the poisoned spores over a 16-hour period. The acetate-induced increase

in the residue fraction was also inhibited. These data show that captan blocked carbohydrate accumulation but not its dissimilation, thus inducing a net loss of carbohydrate.

#### EFFECT OF CAPTAN ON PHOSPHORUS METABOLISM

Only about 70 per cent of the total spore weight was accounted for by the classes of components covered in the previous section (Table III). The remaining 30 per cent consists of nucleic acids, amino and other organic acids, ash, etc. Effects of captan on metabolism of these materials are covered in this and subsequent sections.

An important phase of metabolism is that involving phosphorus turnover, which is responsible in part for the conservation of energy released during oxidations and for the endothermic coupling of small molecules in the synthesis of larger molecules, such as proteins and lipids. Because of the relatively poor incorporation of exogenous phosphate into components of ungerminated spores, it was necessary to analyze captan-induced changes in phosphorus metabolism in terms of endogenous phosphates, i.e., phosphorus-containing compounds already present in the spores. The results of these analyses are shown in Table IV. The inorganic phosphate content remained essentially constant when untreated spores were incubated aerobically for 16 hours with glycerol. This suggests that a relatively steady state existed with regard to phosphorus turnover. When the spores were incubated with various doses of captan ranging from that which had no effect on germination up to one causing 100 per cent inhibition of germination, inorganic phosphate increased in proportion to inhibition of germination. This suggests that captan blocked the reincorporation of inorganic phosphate into organic phosphates, but did not inhibit hydrolysis of organic phosphates.

The source of the increase in inorganic phosphate was revealed by

TABLE IV

EFFECT OF CAPTAN ON PHOSPHORUS LEVELS IN VARIOUS FRACTIONS OF *NEUROSPORA SITOPHILA* CONIDIA INCUBATED WITH GLYCEROL\*

| Amount of captan, mg./g. of spores | Change in phosphorus content, % |         |        |                     | Germination, % |
|------------------------------------|---------------------------------|---------|--------|---------------------|----------------|
|                                    | 2% Perchloric acid extract      |         | Lipids | Ribose nucleic acid |                |
|                                    | Inorganic                       | Organic |        |                     |                |
| 0                                  | - 5                             | -15     | - 7    | +12                 | 100            |
| 2.5                                | 0                               | +30     | 0      | - 6                 | 100            |
| 5.1                                | +11                             | +38     | - 7    | -12                 | 87             |
| 7.6                                | +11                             | +100    | -29    | -19                 | 44             |
| 10.2                               | +22                             | +92     | -36    | -16                 | 4              |
| 12.7                               | +33                             | +92     | -39    | -22                 | 0              |

\* Approximately 400 mg. of glycerol/g. of spores. The incubation period was 16 hours.

simultaneous analyses of other phosphorus containing compounds in the spores (Table IV). It was found that nucleic acid and lipid phosphorus decreased as inorganic phosphate increased. Organic phosphorus in the 2 per cent perchloric acid-soluble fraction almost doubled also. This was probably due to hydrolysis of ribose nucleic acids to nucleotides and of phospholipids to organic phosphate intermediates. The data indicate, therefore, that captan interferes with phosphate turnover, but not with hydrolysis of certain organic phosphates. They indicate further that loss of viability and inhibition of phosphate metabolism occur concomitantly.

Breakdown in functioning of the phosphate cycle was probably a result of inhibition of oxidations, shown in a previous section. The possibility that a lack of suitable phosphate acceptors was responsible is ruled out because of the fact that glucose, acetate, glycerol and other intermediates capable of acting as phosphate acceptors failed to prevent accumulation of phosphorus in inorganic form in poisoned spores.

#### EFFECT OF CAPTAN ON AMINO ACID METABOLISM

It was shown previously that spores of *N. sitophila* metabolize a considerable portion of the free amino acids over a period of 16 hours (7). This was found to be true also in the present study, in which about 70 per cent of the free amino nitrogen disappeared during a 16-hour incubation period (Table V).

TABLE V

EFFECT OF CAPTAN AT A LETHAL DOSE ON THE AMOUNT OF AMINO AND AMIDE NITROGEN IN CONIDIA OF *NEUROSPORA SITOPHILA* AFTER AEROBIC INCUBATION FOR 16 HOURS\*

| Treatment                              | N, mg./g.<br>of spores | Change, % | Captan-induced<br>inhibition, % |
|----------------------------------------|------------------------|-----------|---------------------------------|
| None                                   | 6.3                    | —         | —                               |
| Incubated—no captan                    | 1.9                    | —70       | —                               |
| Incubated with captan<br>(26.4 mg./g.) | 4.5                    | —29       | 41                              |

\* Incubated without added substrate.

When the spores were poisoned with captan, however, the free amino nitrogen remained relatively high, indicating that dissimilation of amino acids was inhibited to a considerable degree. Determinations of individual amino acids by paper chromatography showed that inhibition was not specific for any particular amino acid, but essentially parallel for all of those present.

#### EFFECT OF CAPTAN ON METABOLISM OF OTHER ORGANIC ACIDS

*Keto acids.* The effects of captan at several concentrations on germination and on the levels of  $\alpha$ -ketoglutarate, pyruvate and two unidentified

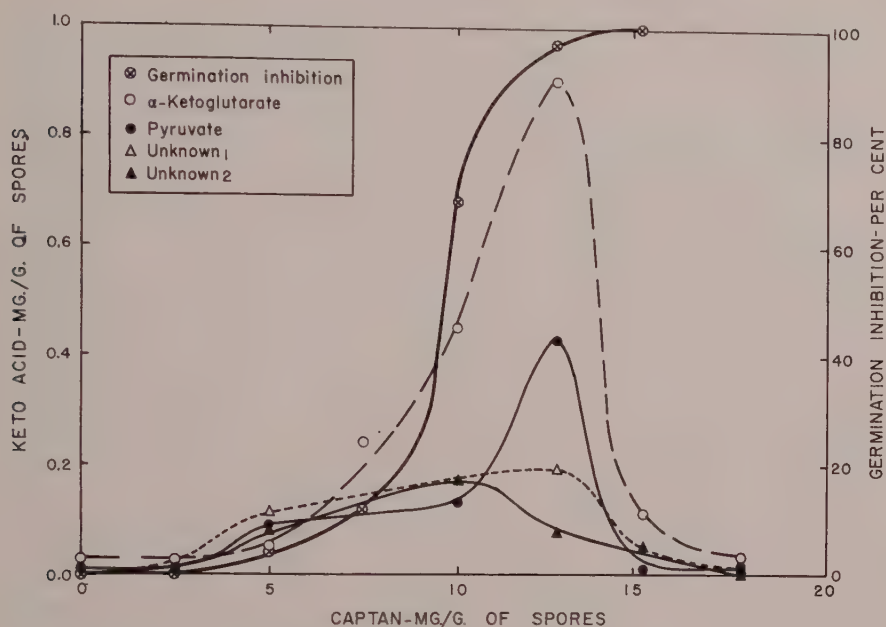


FIGURE 6. Accumulation of keto acids in conidia of *Neurospora sitophila* in relation to germination at several doses of captan.

keto acids in the spores are shown in Figure 6. The four acids accumulated to very high levels in spores receiving the lethal dose of captan, indicating a powerful block in keto acid carboxylase systems. Accumulation of the acids and inhibition of germination increased in proportion to the concentration of captan up to 12.4 mg. of captan/g. of spores. At hyperlethal concentrations, however, the keto acids decreased sharply. This suggests that enzymes responsible for formation of the keto acids from endogenous precursors were inhibited by high concentrations of captan. However, inhibition of keto acid *formation* apparently was secondary in toxicity, since it occurs only at captan concentrations higher than those required to inhibit germination. Inhibition of keto acid *utilization*, on the other hand, was more or less concomitant with inhibition of germination.

*Non-keto acids.* The effects of captan on germination and synthesis of citrate, succinate and malate are shown in Figure 7. Incubation of the spores with sodium acetate resulted in substantial increases in these three acids in untreated spores. In the presence of captan, synthesis of the acids fell off with increasing toxicant concentration and germination declined proportionately.

It should be noted also that the amount of each acid in poisoned spores fell considerably below that in nonincubated spores. This shows that



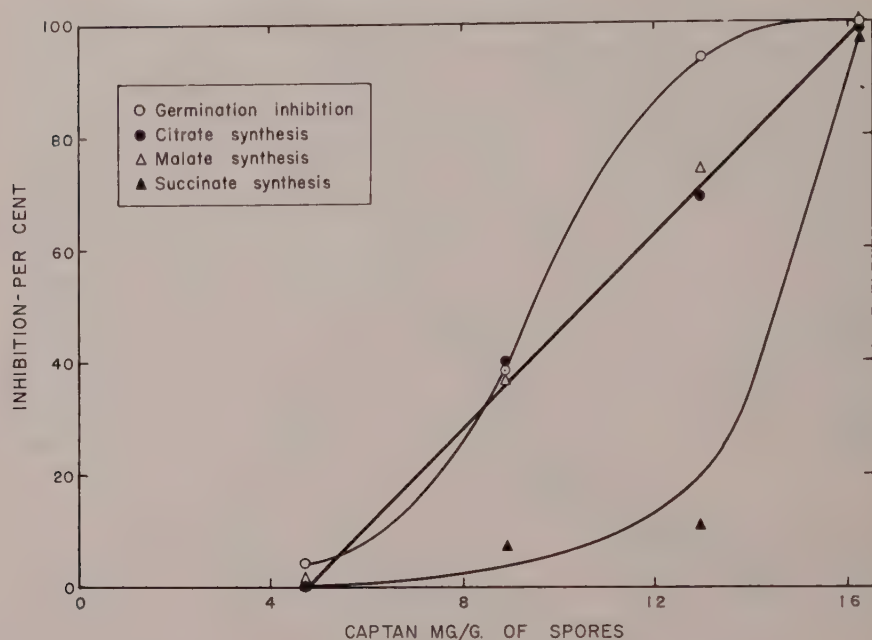


FIGURE 7. Inhibition of citrate synthesis from acetate by conidia of *Neurospora sitophila* in relation to inhibition of germination at several doses of captan.

endogenous acids were metabolized despite the fact that the spores were poisoned. This means, therefore, that the succinoxidase system, cytochrome oxidase, and certain other enzymes in the Krebs cycle remained functional, since blockage of enzymes responsible for dissimilation results in accumulation of the acids, as shown in case of keto acids (Fig. 6), and as found in case of succinic and fumaric acids in studies on other toxicants (unpublished data). Thus, in the metabolism of acetate by way of the Krebs cycle the only enzymes that appear to be affected are  $\alpha$ -ketoglutarate carboxylase (Fig. 6) and one in the 3-step conversion of acetate to citrate. Inhibition of citrate synthesis probably involves inactivation of coenzyme A, since preliminary studies as yet unpublished indicate that coenzyme A is inactivated *in vivo* by captan.

#### DISCUSSION

The present study shows that captan inhibits many metabolic processes in spores of *N. sitophila*. Specifically, the key factors appear to be a number of enzymes in phosphorus metabolism, certain oxidases and dehydrogenases, carboxylases and coenzyme A. A number of reactions were found to proceed in captan-poisoned spores, but they are almost entirely catabolic and cannot support growth. Inhibition of the above processes and enzymes

and inhibition of germination occur concomitantly and are mutually related to the dosage of captan received by the spores. Therefore, the demonstrated effects are at least in part responsible for captan toxicity. Inhibition of other equally important factors not yet known might also contribute to toxicity, but those with which this paper deals are vital to all organisms and the types of inhibition found preclude germination and adaptive formation of by-passes which would permit germination to proceed. It is possible, on the other hand, that some organisms might be relatively resistant to captan because of rapid detoxication of the compound or because of physical or chemical features which tend to exclude it from the cytoplasm.

In reviewing the number of processes affected by captan, the lack of specificity becomes self-evident. Many oxidases and dehydrogenases concerned in endogenous metabolism and in oxidation of exogenous substrates are inactivated. This follows from the fact that the major part of endogenous oxygen uptake and oxidation of all exogenous substrates tested are strongly inhibited. In this respect captan is less discriminatory than dichlone, which was found previously to have little effect on some oxidative processes (7). This indicates a certain degree of individuality in the biological properties of the two toxicants.

On the other hand, there are also a number of similarities. Both compounds induce increases in the R.Q. as the dosage is increased. This is due to more powerful inhibition of reactions incorporating  $O_2$  than of those giving rise to  $CO_2$ . This results in a fundamental change in energy relations of the cell; i.e., progressively more of the potential energy of oxidizable intermediates is dissipated. This is in accord with the fact that phosphorylation is inhibited, since conservation of energy is largely dependent upon phosphorylation.

Although the specific phosphorylating enzymes affected by captan were not determined, accumulation of inorganic phosphate from endogenous sources, which indicates that phosphorylation is blocked, is in agreement with the findings of Duggar *et al.* (2), who showed that captan inhibits yeast hexokinase. The present data indicate that other phosphorylating enzymes are inhibited also, for addition of intermediates requiring other kinases and phosphorylases for phosphorylation shows no tendency to reduce accumulation of inorganic phosphate in poisoned spores.

Inhibition of endogenous metabolism by captan was shown both by inhibition of respiration and by the partial inhibition of  $CO_2$  formation as determined in carbon balance studies. This is in contrast to the effects of dichlone (7), which accelerates some aspects of endogenous metabolism and induces a considerable increase in  $CO_2$  formation from endogenous food reserves. In general, however, the dissimilation of all such reserves proceeds with both toxicants in parallel fashion except for carbohydrate

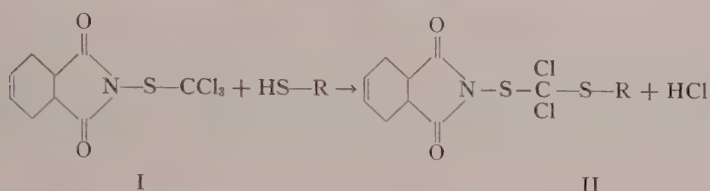
and amino acids. Although both of these components decrease considerably in captan-poisoned spores, they remain at much higher levels than in untreated spores or in spores poisoned with dichlone under the same conditions. This shows that captan partially inhibits enzymes concerned in carbohydrate and amino acid dissimilation whereas dichlone does not.

Results from experiments on keto acid metabolism are in accord with earlier findings of Hochstein and Cox (3) and Silber (9), who found that pyruvate carboxylase is inhibited by captan. In addition, the present data show that certain other keto acids, including  $\alpha$ -ketoglutarate, accumulate and that keto acid accumulation coincides with inhibition of germination. Hochstein and Cox were able to reverse inhibition of pyruvate carboxylase *in vitro* by adding cocarboxylase to the system, but they were unable to reverse inhibition of cellular growth. This suggests that inhibition of pyruvate carboxylase is due to inactivation of cocarboxylase, in which case other carboxylases requiring cocarboxylase would be inhibited also. However, since this is only one factor in inhibition of growth, it can be anticipated that inhibition of growth cannot be reversed by addition of components of carboxylase systems.

Inhibition of the synthesis of citrate from acetate shows that entrance into the Krebs cycle through citrogenase is blocked because of inactivation of some factor in the pathway from acetate to citrate. Preliminary unpublished data show that this factor is coenzyme A. Reaction of captan with coenzyme A is also indicated by the data of Lukens and Sisler (5), which show that coenzyme A and a number of other thiols are effective in reducing the toxicity of captan when added to cells before or along with captan.

Another aspect of fundamental interest in captan action is the nature of the interaction between the toxicant and the enzymes and coenzymes it inactivates. Lukens and Sisler (5) have made an unusually thorough study of the reactions of captan with thiols, particularly cysteine. They found that the reaction results in formation of a number of by-products. However, of the thiols tested only cysteine and thioglycerol gave rise to a product absorbing strongly in the ultraviolet in the region between 2700 and 2800 Å. This suggests that the reaction between cysteine and captan, or thiophosgene, as they postulate, is a special case rather than a general reaction. The present data also indicate this, for it was shown that toxicity is correlated with the amount of captan absorbed by the spores, that an excess of captan in the ambient solution is necessary to kill the spores, and that when all available captan is absorbed metabolism and viability of the spores tend to recover. This is inconsistent with the idea that thiophosgene is the "ultimate toxicant," because thiophosgene would be formed intracellularly even after all of the external captan was absorbed. Furthermore, Lukens and Sisler show that thiophosgene is formed when

cysteine and captan react; i.e., the *reaction* gives rise to thiophosgene. The biological data suggest that it is the reaction between the toxicant and the thiol that constitutes the toxic reaction. It is suggested, therefore, that captan toxicity results from a straightforward substitution as follows:



where R—SH represents any compound in the cells containing a free, unprotected thiol group. The product, II, undoubtedly decomposes spontaneously in several directions to yield products of the kinds characterized by Lukens and Sisler. Decomposition, however, is probably secondary because inactivation of R—SH would occur when the initial reaction takes place.

A further point, however, is that decomposition of compound II could proceed in such a manner as to release R—SH intact, with simultaneous formation of tetrahydrophthalimide and thiophosgene. This means, of course, that R—SH, if it were an enzyme or coenzyme, would be free to participate in its normal capacity in metabolism. In this event the only harm done to the spore would be retardation of certain reactions, unless thiophosgene were the "ultimate toxicant," or unless an excess of captan were present to combine again with the liberated molecule. The biological data suggest that R—SH is sometimes liberated intact, for inhibition of metabolic functions and germination recover to some extent unless excess captan is present. Obviously, however, since only a portion of metabolism recovers, some enzymes and coenzymes must be permanently inactivated, possibly by decomposition of the captan—S—R intermediate in such a way as to split off the thiol group.

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# *m*-NITRO- AND *m*-TRIFLUOROMETHYL-ARYL ACIDS AS PLANT GROWTH REGULATORS

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## SUMMARY

Sixteen compounds with a *meta* directing group at the *meta* position of the benzene ring as related to phenoxyacetic acid and *N*-phenylglycine were prepared and evaluated for cell elongation activity in the tomato leaf curvature test. The trifluoromethyl group placed in the *meta* position functioned as an activity-enhancing group like the nitro group in the same position.

## INTRODUCTION

It is well known that the halogen atom in the *ortho* position of 2,4-dichlorophenoxyacetic acid can be replaced with a methyl group without a noticeable decrease in plant growth-regulating activity (13), but not with a nitro group. In the case of 3,4-dichlorophenoxyacetic acid, replacement of a chlorine atom with a methyl group in the *meta* position causes a decrease in cell elongation activity (13). A similar reduction in activity occurs in *N*-(3,4-dichlorophenyl)glycine (14), but in this case a chlorine atom in the *meta* position could be replaced with a nitro group without a noticeable decrease in activity. Zimmerman (19) in 1943 reported *m*-nitrophenoxyacetic acid as active. These results and the results of other workers (17, 18) suggest that the introduction of a *meta* directing group into the *meta* position of aryloxyacetic acid or *N*-arylglycine might increase the physiological activity of the compound.

The present report describes the experiments employed along these lines. Sixteen compounds with a *meta* directing group, such as nitro, trifluoromethyl or carboxyl, in the *meta* position of the benzene ring have been prepared and tested for physiological activity on tomato plants.

## PREPARATION OF COMPOUNDS

The compounds and their properties are listed in Table I. The preparation of the series of compounds leading to *N*-(3-trifluoromethyl-4-chlorophenyl)glycine will first be described in detail.

*N*-(3-Trifluoromethyl-4-chlorophenyl)glycinonitrile. A mixture of 49 g. (0.25 mole) of 3-trifluoromethyl-4-chloroaniline (3) and 125 ml. of 3*M* formaldehyde-sodium bisulfite solution (0.38 mole) was heated under re-

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TABLE I  
PROPERTIES OF *m*-SUBSTITUTED ARYL ACIDS

| Compounds                                                       | Yield  | M.p., ° C.,<br>uncorrected | Reported m.p.,<br>° C., and<br>references | Formula           | Carbon * |       | Hydrogen |       | Nitrogen ** |         |
|-----------------------------------------------------------------|--------|----------------------------|-------------------------------------------|-------------------|----------|-------|----------|-------|-------------|---------|
|                                                                 |        |                            |                                           |                   | Calcd.   | Found | Calcd.   | Found | Calcd.      | Found   |
| <i>N</i> -( <i>m</i> -(Trifluoromethyl)phenyl)glycine           | 24.1 † | 128–130 dec.               | 117.5 (8)                                 | $C_9H_7ClF_3NO_2$ | 42.62    | 42.71 | 2.78     | 2.09  | 11.09       | 10.44   |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycine             | 67.0   | 130–131 dec.               |                                           | $C_9H_5ClF_3N_2O$ |          |       |          |       | 11.94       | 11.61   |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycinamide         | 41.7   | 109–111                    |                                           |                   |          |       |          |       |             |         |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycinonitrile      | 72.5   | 76–78                      |                                           | $C_9H_6ClF_3N_2$  |          |       |          |       |             |         |
| <i>m</i> -(Trifluoromethyl)phenoxyacetic acid                   | 53.1   | 94–96                      | 93.5–94 (11)                              | $C_8H_8N_3O_3$    |          |       |          |       | 21.53       | 21.4 †† |
| <i>N</i> -( <i>m</i> -Nitrophenyl)glycine                       | 53.0 † | 160–162 dec.               | 158–159 (2)                               | $C_8H_{11}N_3O_3$ | 51.67    | 51.01 | 5.31     | 4.95  |             |         |
| <i>N</i> -( <i>m</i> -Nitrophenyl)-glycinamide                  | 39.5 † | 163–165                    |                                           |                   |          |       |          |       |             |         |
| <i>N</i> -( <i>m</i> -Nitrophenyl)- <i>N</i> -methylglycinamide | 95.8   | 160–161                    |                                           |                   |          |       |          |       |             |         |
| <i>m</i> -Nitrophenoxyacetic acid                               | 76.2   | 151–152                    | 151 (10)                                  |                   |          |       |          |       |             |         |
| <i>m</i> -Nitrophenoxyacetamide                                 | 69.5   | 189–190                    | 178.5 (10)                                |                   |          |       |          |       |             |         |
| $\alpha$ -( <i>m</i> -Nitrophenoxy)-propionic acid              | 42.4   | 113–114                    | 107–110 (1)                               |                   |          |       |          |       |             |         |
| 3,5-Dinitrophenoxyacetic acid                                   | 62.2   | 165–167                    | 207 † (12)                                | $C_8H_6N_2O_7$    | 39.68    | 40.07 | 2.50     | 2.55  |             |         |
| <i>m</i> -Carboxyphenoxyacetic acid                             | 89.4   | 208.5–209                  | 206–207 (9)                               |                   |          |       |          |       |             |         |
| <i>m</i> -Nitrophenylacetic acid                                |        | 118–119                    | 117 (4)                                   |                   |          |       |          |       |             |         |
| <i>N</i> -(3,4-Dichlorophenyl)-glycine                          |        | 128–129 dec.               | 128–129 (14)                              |                   |          |       |          |       |             |         |
| <i>N</i> -(3,4-Dichlorophenyl)-glycinamide                      |        | 139–140                    | 139–139.5 (14)                            |                   |          |       |          |       |             |         |

\* Analyses made by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y., excepting the nitrogen determination of *N*-(*m*-nitrophenyl)-*N*-methylglycinamide.

\*\* By Dumas' method.

† The yields have been calculated on the basis of anilines.

†† By modified semi-micro Kjeldahl (15).

‡ Apparently not the same compound, see text.

flux. After 30 to 45 minutes of refluxing, the mixture became clear. The heating was continued further for 30 minutes. After cooling, it solidified to a crystal mass. The crystals, which consisted of almost pure sodium *N*-(3-trifluoromethyl-4-chlorophenyl)-aminomethanesulfonate, were collected on a Buechner funnel with suction, washed twice with a small amount of cold ethanol and dried. The crystal powder was redissolved in 125 ml. of water. A solution of 20 g. of potassium cyanide in 50 ml. of water was added and the resulting solution was refluxed for one hour. The crystals which separated on cooling were filtered with suction and dried in a stream of air under suction for a few days, m.p. 76° to 78° C. The analytical sample was recrystallized twice from ethanol and gave light yellow prisms melting at 76° to 78° C.

*N*-(3-Trifluoromethyl-4-chlorophenyl)glycinamide. Into 6 ml. of concentrated sulfuric acid 4.7 g. (0.02 mole) of the above crude nitrile were added carefully, with stirring. The temperature was kept below 30° C. during the addition. After being warmed up to 60° C. for several minutes, then left at room temperature for 30 minutes, the reaction mixture was diluted with 100 ml. of water with ice cooling and then neutralized with 20 per cent aqueous sodium hydroxide to separate the amide. One recrystallization of the crude product from hot water gave 2.1 g. of white prisms melting at 109° to 111° C.

*N*-(3-Trifluoromethyl-4-chlorophenyl)glycine. The mixture of 11.7 g. (0.05 mole) of the above nitrile and 30 ml. of concentrated hydrochloric acid (37 per cent) was placed in a 1000-ml. evaporating dish and heated on a steam bath for two hours. The resulting mass was neutralized with 20 per cent aqueous sodium hydroxide and then was diluted with 500 ml. of water. After being adjusted to a pH of 6.0, it was treated with Norit-A. The free acid was precipitated by acidifying the filtered solution to a little lower than pH 4.0 with a diluted hydrochloric acid (1:1). Yield of a crude product was 8.5 g., m.p. 130° to 131° C., after one recrystallization from hot water.

*Other N-aryl-glycines.* The other aryl-glycines were prepared similarly to the above or by the methods indicated by the literature references in Table I. An *N*-methyl-*N*-aryl-glycine derivative was made by the following method.

*N*-Methyl-*N*-(*m*-nitrophenyl)glycinamide. To a suspension of *N*-(*m*-nitrophenyl)glycinamide (3.9 g., 0.02 mole) in 25 ml. of acetone 2.8 g. (0.022 mole) of dimethyl sulfate were added dropwise, with stirring. The mixed solution was refluxed on a steam bath for one hour. After adding 5 ml. of 10 per cent aqueous sodium hydroxide and 100 ml. of water, the reaction mixture was kept warm for several minutes. The yield was almost theoretical, m.p. 160° to 161° C., after three recrystallizations from hot water.



*Aryloxy acids.* The aryloxyacetic acids and their amides were prepared through the usual reaction of sodium phenolate and sodium chloroacetate or chloroacetamide.

$\alpha$ -Bromopropionic acid and *m*-nitrophenol were used to prepare  $\alpha$ -*m*-nitrophenoxypropionic acid. This latter compound was indicated by Bischoff (1) to be unstable to air; however, no change was observed in this laboratory after keeping the compound for several months over calcium chloride in a dessicator.

*3,5-Dinitrophenoxyacetic acid.* 3,5-Dinitrophenol was prepared from 1,3,5-trinitrobenzene through 3,5-dinitroanisole in the same manner as the description in the literature (5). Aqueous potassium 3,5-dinitrophenolate solution was made by adding 3.7 g. (0.02 mole) of 3,5-dinitrophenol to a solution of potassium hydroxide (1.1 g., 0.02 mole) in 5 ml. of water. A solution of potassium chloroacetate was made by adding 8 g. (0.08 mole) of potassium bicarbonate to a solution of chloroacetic acid (7.6 g., 0.08 mole) in 20 ml. of water. These two solutions were mixed and heated for two hours under reflux on a steam bath, yielding 3.0 g. of a crude product with a melting point of 163° to 166° C. which was raised to 165° to 167° C. after recrystallizations first from water, then from benzene. Reverdin and Bucky (12) through a series of different reactions had obtained a compound melting at 207° C. which has been assumed to be 3,5-dinitrophenoxyacetic acid but which would now appear questionable.

#### TESTING FOR PHYSIOLOGICAL ACTIVITY

The compounds were tested for cell elongation activity on tomato (*Lycopersicon esculentum* Mill.) plants that had four moderately developed leaves.

Solutions of the compounds were applied as sprays, as a 0.01-ml. application to the terminal leaflet of the third leaf or by the addition of 25 ml. to the soil surface as previously described (6, 20) and the threshold concentration, expressed in w/v per cent, required for a response was determined.

#### RESULTS AND DISCUSSION

The results of the tests are given in Table II. For comparative results with 2,4-D (2,4-dichlorophenoxyacetic acid) see the paper by Hitchcock and Zimmerman (6).

Ten of the 16 compounds synthesized were active for inducing epinasty of tomato leaves, and three were inactive (Table II). *N*-(*m*-Nitrophenyl)-*N*-methylglycinamide, *m*-nitrophenoxyacetamide and  $\alpha$ -(*m*-nitrophenoxy)-propionic acid were inactive when sprayed on the foliage but were active when applied to the soil. This suggests that these three chemicals, of low solubility in water, might be absorbed and transferred to the active site of the plant more readily through roots than through leaves, or a chemical

TABLE II  
RELATIVE ACTIVITY OF *m*-SUBSTITUTED ARYL ACIDS IN  
TOMATO LEAF CURVATURE TEST

| Compound                                                                                     | Threshold<br>concn. of solution, w/v % |
|----------------------------------------------------------------------------------------------|----------------------------------------|
| A. Active when applied as a solution (0.01 ml.) to the terminal<br>leaflet of the third leaf |                                        |
| <i>N</i> -(3,4-Dichlorophenyl)glycine                                                        | 0.03                                   |
| <i>N</i> -(3,4-Dichlorophenyl)glycinamide                                                    | 0.03                                   |
| <i>N</i> -( <i>m</i> -Nitrophenyl)glycine                                                    | 0.13                                   |
| <i>N</i> -( <i>m</i> -Nitrophenyl)glycinamide*                                               | 0.25                                   |
| <i>m</i> -Nitrophenylacetic acid                                                             | 0.06**                                 |
| <i>m</i> -Nitrophenoxyacetic acid                                                            | (0.13†)                                |
| <i>N</i> -[( <i>m</i> -Trifluoromethyl)phenyl]glycine                                        | 0.13                                   |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycine*                                         | 0.50                                   |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycinamide*                                     | 0.50                                   |
| <i>m</i> -(Trifluoromethyl)phenoxyacetic acid                                                | 0.03                                   |
| B. Active when added to soil†† but not active on foliage                                     |                                        |
| <i>N</i> -( <i>m</i> -Nitrophenyl)- <i>N</i> -methylglycinamide*                             | 0.015                                  |
| <i>m</i> -Nitrophenoxyacetamide                                                              | 0.006‡                                 |
| $\alpha$ -( <i>m</i> -Nitrophenoxy)propionic acid                                            | 0.015                                  |
| C. Not active                                                                                |                                        |
| <i>N</i> -(3-Trifluoromethyl-4-chlorophenyl)glycynitrile*                                    | —                                      |
| 3,5-Dinitrophenoxyacetic acid                                                                | —                                      |
| <i>m</i> -Carboxyphenoxyacetic acid                                                          | —                                      |

\* New compounds.

\*\* Active also for modification.

† Active as a 0.13% spray but not as a 0.01-ml. application of a 1.0% solution to one leaflet.

†† Twenty-five milliliters of solution added to the soil surface.

‡ Not entirely soluble.

reaction might have taken place in the soil to produce active components.

The author has inferred in a preceding paper (16) that *N*-methylation of *N*-aryl-glycine tends to reduce activity. The present result with *N*-(*m*-nitrophenyl)glycinamide and its *N*-methyl derivative also substantiates the above inference.

When either the trifluoromethyl or the nitro group was present in the *meta* position of the benzene ring there was an enhancement of activity. However, when a carboxyl group was present in this position no enhancement of activity was produced.

In view of the activity-enhancing character of a nitro group placed in the *meta* position of the benzene ring, it was considered that 3,5-dinitrophenoxyacetic acid might be active; however, the introduction of a second *meta* nitro group rendered the compound physiologically inactive. This may be in accord with the theory of Leaper and Bishop (7) that two unsubstituted positions *para* to each other are necessary for activity.

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# $\alpha$ -SUBSTITUTED ARYL-ACETIC ACIDS AS PLANT GROWTH REGULATORS

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## SUMMARY

Eighteen compounds including seven ring-substituted phenylglycolic acids, the corresponding *O*-methyl derivatives and four related compounds were prepared and tested for physiological activity on tomato plants.

*O*-Methylated derivatives were usually more active than the unmethylated compounds, but the reverse was true in the case of  $\alpha$ -methoxy-(2-chlorophenyl)-acetic acid. Chlorine atoms introduced into the benzene ring tended to enhance the activity depending upon the number and the position of the substitution. Among the compounds so far tested  $\alpha$ -methoxy-(3,4-dichlorophenyl)acetic acid was the most active.

## INTRODUCTION

A large number of aryl-acetic acids and their ring substitution products are known to be active plant growth regulators (8). In regard to substituted aryl-acetic acids bearing a substituent on the  $\alpha$ -carbon atom of the acetic acid moiety, particularly a more polar substituent, much less is known. None of the  $\alpha$ -hydroxy-aryl-acetic acids other than  $\alpha$ -naphthylglycolic acid (18) and phenylglycolic (mandelic) acid (14) had been investigated in this respect. The latter is physiologically inactive or of questionable activity (17). The probable cause of its inactivity is the presence of a highly hydrophylic radical on the side chain.

On the basis of this assumption, a more lipophylic radical on the side chain of mandelic acid should result in a physiologically active compound. A clue is apparently given by the recent findings of Mitchell *et al.* (9, 10) that  $\alpha$ -methoxyphenylacetic acid is quite active in modifying vegetative growth of plants as well as in promoting the growth of axillary shoots of tomato plants. The importance of the chlorine substitution in the benzene ring has also been suggested. Reeve and Pickert (12) prepared 2,4-dichloro and 3,4-dichloro  $\alpha$ -methoxyphenylacetic acids. The preparation and testing of mono-substituted derivatives of this series still seem of importance from the standpoint of the relationship of chemical structure to physiological activity.

The purpose of the present work is to compare the influence of ring substitution on the plant growth-promoting activity of the above-men-

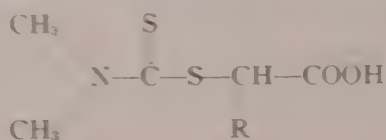
<sup>1</sup> The Ohara Institute for Agricultural Biology, Okayama University, Kurasaki, Japan. Research conducted at Boyce Thompson Institute for Plant Research under a post-doctoral fellowship of The Rockefeller Foundation (1957-1958). The writer wishes to express his thanks to Dr. George L. McNew, Managing Director of Boyce Thompson Institute, and to Dr. A. E. Hitchcock and Dr. E. A. Prill for assistance in the preparation of the manuscript. Physiological activity of the chemicals was tested by the late Dr. P. W. Zimmerman.

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thyl analogues including the  $\alpha$ -naphthyl derivative. Fourteen compounds of the above-mentioned series, including three that are new, were prepared and tested for cell elongation activity on tomato (*Lycopersicon esculentum* Mill.) plants as previously described (4, 20). Five other  $\alpha$ -substituted aryl-acetic acids were also prepared and tested for physiological activity.

These included two dithiocarbamates of the formula:



wherein R denotes the phenyl and the 2,4-dichlorophenyl radicals, respectively, which may be of special interest in that they combine the structural features of both the active dithiocarbamates recently reported by van der Kerk *et al.* (19) and the  $\alpha$ -substituted aryl-acetic acids.

#### PREPARATION OF COMPOUNDS

With the exception of the *m*-nitro derivatives, the aryl-glycolic acids and the  $\alpha$ -methoxy-aryl-acetic acids were prepared by adaptation of the method of Reeve and co-workers (11, 12). The barium salt of  $\alpha$ -sulfo-phenylacetic acid was prepared according to Brust (21). The reactions involving cyanides were performed in a well ventilated hood.

*m*-Nitrophenylglycolic acid. This was prepared by an adaptation of the method used by Lapworth (6) for mandelic acid. A solution of 21.5 g. (0.33 mole) of potassium cyanide in 25 ml. of water was placed in a 2000-ml. three-neck flask equipped with a mechanical stirrer, reflux condenser and dropping funnel. A solution of 45.3 g. (0.3 mole) of *m*-nitrobenzaldehyde in 300 ml. of ether was cautiously added with stirring. The stirring was continued for half an hour and then the aqueous layer was removed and disposed of in a safe manner. After addition of a solution of 30 ml. of concentrated hydrochloric acid in 400 ml. of ethanol to the ethereal solution, the mixture was treated for about 12 hours with a current of HCl gas. After removal of the solvents under reduced pressure, the residue was transferred to an evaporating dish and taken to dryness on a steam bath. The residue was then taken up in 10 per cent NaOH solution and decolorized with Norit-A. Acidification with dilute HCl precipitated the crude product. Recrystallization first from water, then from benzene, gave a pure sample melting at 131° to 132° C.

Ethyl 2,4-dichlorophenylglycolate. A solution of 22.2 g. (0.1 mole) of 2,4-dichlorophenylglycolic acid in 50 ml. of absolute ethanol was refluxed for one hour with dry HCl gas passed through the reaction mixture. By

diluting the mixture with 500 ml. of water an oily product was precipitated. It was extracted with ether, and the ethereal extract, after being dried with anhydrous sodium sulfate, was fractionally distilled at a diminished pressure. A fraction distilling at 178° to 179° C. at 13 mm. was collected, which solidified at room temperature. The yield was 17.8 g. (71.5 per cent) of a product melting at 40° to 41° C. after one recrystallization from petroleum ether. *Anal*: Calcd. for  $C_{10}H_{10}Cl_2O_3$ : C, 48.22. H, 4.05. Found: C, 48.90. H, 4.26.

This ester had also been prepared by another method by Barthel *et al.* (1).

*Ethyl  $\alpha,2,4$ -trichlorophenylacetate*. To a mixture of 12.5 g. (0.05 mole) of ethyl 2,4-dichlorophenylglycolate with 4.4 g. (0.055 mole) of pyridine, which was cooled in an ice bath, 6.6 g. (0.055 mole) of thionyl chloride were added dropwise. The reaction mixture was heated on a boiling water bath for an additional one hour. After it was washed with hydrochloric acid (1:1), then with 10 per cent aqueous sodium carbonate, and finally with water, the ester was extracted into 100 ml. of benzene and dried with sodium sulfate. After evaporation of the solvent, it was fractionally distilled under reduced pressure. A fraction consisting of a colorless oil weighing 11.8 g. (88.1 per cent yield) was collected at 163° to 165° C./11 mm.

*S-(2,4-Dichloro- $\alpha$ -carboxybenzyl) N,N-dimethyldithiocarbamate*. Carbon disulfide (1.7 g., 0.022 mole) was added to a mixture consisting of 4 g. (0.022 mole) of aqueous dimethylamine (25 per cent) and 5 ml. (0.044 mole) of aqueous potassium hydroxide (35 per cent) with vigorous stirring in an ice bath. The stirring was continued for several hours. To the resulting clear potassium *N,N*-dithiocarbamate solution (3), ethyl  $\alpha,2,4$ -trichlorophenylacetate (5.3 g., 0.02 mole) was added with stirring at room temperature. The temperature of the reaction mixture increased up to 60° C. by itself. After being kept in a water bath at 60° to 70° C. for 30 minutes, it was diluted with 75 ml. of water and filtered. On acidifying the solution with dilute hydrochloric acid (1:1) and adjusting the pH to 3.0 to 4.0, a resinous mass was separated. One recrystallization from dilute aqueous ethanol gave 2.3 g. (35.6 per cent) of almost pure sample, melting at 162° to 163° C.

*S-( $\alpha$ -Carboxybenzyl) N,N-dimethyldithiocarbamate* was prepared similarly.

#### TESTING FOR PHYSIOLOGICAL ACTIVITY

The compounds were tested for cell elongation and leaf modification activities on tomato (*Lycopersicon esculentum* Mill.) plants that had four moderately developed leaves.

Solutions of the compounds were applied as a 0.01-ml. application to the terminal leaflet of the third leaf as previously described (4, 20) and

the threshold concentration, expressed in w/v per cent, required for response was determined.

### RESULTS AND DISCUSSION

The relative activities of the 18 compounds are shown in Tables I and II. For comparative results with 2,4-D (2,4-dichlorophenoxyacetic acid) see the paper by Hitchcock and Zimmerman (4). Both  $\alpha$ -(3-chlorophenyl)-glycolic acid and  $\alpha$ -(4-chlorophenyl)glycolic acid were inactive, while the corresponding *O*-methyl derivatives were active. This is in accord with the relationship between phenylglycolic acid and  $\alpha$ -methoxyphenylacetic acid with respect to physiological activity. Several unmethylated compounds, such as  $\alpha$ -(2-chlorophenyl)-,  $\alpha$ -(2,4-dichlorophenyl)- and  $\alpha$ -(3,4-dichlorophenyl)glycolic acids, were of low activity, and  $\alpha$ -(1-naphthyl)glycolic acid was inactive, thus demonstrating the importance of ring substitution as suggested by Mitchell and Preston (9), but the *O*-methylation of these compounds is important for their physiological activity since most of the *O*-methylated aryl-glycolic acids so far tested were more active than the unmethylated ones. In the case of 2-chlorophenyl compounds, however, the situation was reversed. Exceptions were also noted in other types of compounds; for example, a nitro group in the *meta* position of the benzene ring often functions as an activity-enhancing group (13, 19), but *m*-nitrophenylglycolic acid and its *O*-methyl derivative were inactive.

In addition to the epinastic response, three  $\alpha$ -methoxy chlorinated phenylacetic acids, namely, the 3-chloro-, 2,4-dichloro- and 3,4-dichloro-, caused modification of leaves when 0.01 ml. of the solution was applied to the terminal leaflet of the third leaf. The threshold concentrations of the solution required for modification were, respectively, 0.25, 0.25 and 0.06 w/v per cent.

It is evident from the present results that the introduction of one chlorine atom into the benzene ring of these compounds increases the activity only slightly, or not at all, depending upon the position of substitution. Two chlorine atoms introduced into the 2,4- and 3,4-positions exerted more effect in enhancing activity, and  $\alpha$ -methoxy-(3,4-dichlorophenyl)-acetic acid was found most active among them.

Several other  $\alpha$ -substituted phenylacetic acids (Table II) did not show activity excepting possibly ethyl  $\alpha$ ,2,4-trichlorophenylacetate. Further assessment of the last compound is necessary because of its low solubility in water.

TABLE I  
PROPERTIES OF ARYL-GLYCOLIC ACIDS AND  $\alpha$ -METHOXY-ARYL-ACETIC ACIDS

| Compound                                             | Yield* | M.p.,<br>uncor-<br>rected,<br>°C. | Reported m.p.,<br>°C., and<br>references | Formula         | Carbon** |       | Hydrogen |       | Activity<br>for cell<br>elongation,<br>threshold<br>concn. of<br>solution, w/v%† |
|------------------------------------------------------|--------|-----------------------------------|------------------------------------------|-----------------|----------|-------|----------|-------|----------------------------------------------------------------------------------|
|                                                      |        |                                   |                                          |                 | Calcd.   | Found | Calcd.   | Found |                                                                                  |
| $\alpha$ -(1-Naphthyl)glycolic acid                  | 29.5   | 191-193                           | 98-99 (7)                                |                 |          |       |          |       | Not active                                                                       |
| $\alpha$ -Methoxy-(1-naphthyl)acetic acid            | 37.0   | 151-153                           | 149-150 (15)                             |                 |          |       |          |       | 1.0                                                                              |
| $\alpha$ -(2-Chlorophenyl)glycolic acid              | 80.4   | 93-95                             | 85-85.5 (5)                              |                 |          |       |          |       | 1.0                                                                              |
| $\alpha$ -Methoxy-(2-chlorophenyl)-acetic acid†      | 30.0   | 85-85.5                           |                                          | $C_8H_9ClO_3$   | 53.88    | 53.63 | 4.52     | 4.55  | Not active                                                                       |
| $\alpha$ -(3-Chlorophenyl)glycolic acid              | 53.3   | 99-100                            | 115-115.5 (5)                            |                 |          |       |          |       | Not active                                                                       |
| $\alpha$ -Methoxy-(3-chlorophenyl)-acetic acid†, ††  | 54.0   | Liquid                            |                                          | $C_8H_9ClO_3$   | 53.88    | 53.66 | 4.52     | 4.59  | 0.5                                                                              |
| $\alpha$ -(4-Chlorophenyl)glycolic acid              | 63.1   | 122-124                           | 120.5-121 (5)                            |                 |          |       |          |       | Not active                                                                       |
| $\alpha$ -Methoxy-(4-chlorophenyl)-acetic acid†      | 63.0   | 83-85                             |                                          | $C_8H_9ClO_3$   | 53.88    | 54.41 | 4.52     | 4.52  | 0.5                                                                              |
| $\alpha$ -(3-Nitrophenyl)glycolic acid               | 62.2   | 129-130                           | 119 120                                  |                 |          |       |          |       | Not active                                                                       |
| $\alpha$ -Methoxy-(3-nitrophenyl)-acetic acid†       | 50.0   | 146-147                           |                                          | $C_8H_9NO_5$    | 51.19    | 51.64 | 4.30     | 4.37  | Not active                                                                       |
| $\alpha$ -(2,4-Dichlorophenyl)glycolic acid          | 53.9   | 127-128                           | 120-121 (12)                             |                 |          |       |          |       | 1.0                                                                              |
| $\alpha$ -Methoxy-(2,4-dichlorophenyl)-acetic acid†† | 61.8   | 114-116                           | 115 (12)                                 |                 |          |       |          |       | 0.25                                                                             |
| $\alpha$ -(3,4-Dichlorophenyl)glycolic acid          | 62.5   | 120-121                           | Liquid (12)                              | $C_8H_6Cl_2O_3$ | 43.46    | 43.97 | 2.74     | 2.87  | 1.0                                                                              |
| $\alpha$ -Methoxy-(3,4-dichlorophenyl)-acetic acid†† | 61.8   | 88-91                             | 89 (12)                                  |                 |          |       |          |       | 0.03                                                                             |

\* Calculated on the basis of aromatic aldehydes for aryl-glycolic acids and on the basis of aryl-glycolic acids for  $\alpha$ -methoxy-aryl-acetic acids, respectively.

\*\* Analyses were made by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

† New compounds.

†† Active for modification of leaves.

‡ Based on tomato leaf curvature test when 0.01 ml. of a solution is applied to the terminal leaflet of the third leaf.



TABLE II  
PROPERTIES OF SEVERAL  $\alpha$ -SUBSTITUTED PHENYLACETIC ACIDS\*

| Compound                                                                                                                             | Yield        | M.p.,<br>uncorr.,<br>° C.              | Formula                                                                  | Carbon |       | Hydrogen |       | Nitrogen     |              |
|--------------------------------------------------------------------------------------------------------------------------------------|--------------|----------------------------------------|--------------------------------------------------------------------------|--------|-------|----------|-------|--------------|--------------|
|                                                                                                                                      |              |                                        |                                                                          | Calcd. | Found | Calcd.   | Found | Calcd.       | Found**      |
| Barium salt of $\alpha$ -sulfophenylacetic acid<br><i>S</i> -(2,4-Dichloro- $\alpha$ -carboxybenzyl)<br><i>N,N</i> -dithiocarbamate† | 74.0         | 162-163                                | $C_8H_6O_6S_2Ba \cdot H_2O$                                              |        |       |          |       |              |              |
| <i>S</i> -( $\alpha$ -Carboxybenzyl)<br><i>N,N</i> -dithiocarbamate†                                                                 | 35.6<br>88.9 | 178-180<br>(B.p.<br>164-165/11<br>mm.) | $C_{11}H_{13}NO_3S_2$<br>$C_{11}H_{11}Cl_2NO_3S_2$<br>$C_{10}H_9Cl_3O_2$ |        |       |          |       | 5.49<br>4.32 | 5.27<br>4.02 |
| $\alpha$ ,2,4-Trichlorophenylacetic ethyl ester†                                                                                     |              |                                        |                                                                          | 44.89  | 45.37 | 3.39     | 3.45  |              |              |

\* Physiologically inactive except possibly  $\alpha$ ,2,4-trichlorophenylacetic ethyl ester which was indefinite because of its very low solubility in water.

\*\* By Dumas' method.

† New compounds.

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# COMPARATIVE STABILITIES OF DYRENE, 1-FLUORO-2,4-DINITROBENZENE, DICHLONE AND CAPTAN IN A SILT LOAM SOIL<sup>1</sup>

H. P. BURCHFIELD

## SUMMARY

The stability of 4 organic fungicides in a silt loam soil decreased in the order: captan > dichlone > 1-fluoro-2,4-dinitrobenzene > Dyrene. Based on colorimetric analysis of reactive halogen, one-half of the Dyrene added to moist soil disappeared within 0.5 day while 3 to 4 days were required for the deterioration of the same proportion of captan. Breakdown of the fungicides was much slower in air-dried soil. Stability in soil could not be correlated with hydrolysis rate in aqueous buffer solutions. For example, captan survived 7 times longer than Dyrene in moist soil while Dyrene was about 200 times more stable in aqueous buffer at pH 7 than captan. This apparent anomaly may arise from the fact that Dyrene has moderately high capacity for reacting with amino groups while captan is more sulfhydryl-specific. Thus the concept of group specificity may be important in interpreting the interactions of toxicants with their environments, as well as in correlating mechanisms of biocidal action.

## INTRODUCTION

Many of the organic chemicals used for the control of fungus diseases and nematodes are highly reactive with amino, sulfhydryl and other functional groups found in amino acids, peptides and proteins (7, 8, 9, 10, 11). This may account in part for their biological activities, but it could also result in their rapid attrition in media rich in organic matter such as soils. Recently colorimetric procedures have been developed for the analysis of a number of pesticides containing reactive halogen atoms (5). These tests do not detect their reaction or hydrolysis products, so it is possible to compare deterioration rates of these materials in soils and in aqueous solutions.

In this paper the stabilities of captan, dichlone, Dyrene, and 1-fluoro-2,4-dinitrobenzene are compared in pH 7 buffer and in silt loam soil and the results are discussed in terms of their known chemical and physical properties. Originally it was expected that similar studies would be made on other soil types including sterile soil and sand, but this project was terminated before this could be accomplished.

## MATERIALS AND METHODS

### CHEMICALS

Captan was added to soil in the form of a pyrophilite dust containing 5 per cent *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide. This was prepared by grinding 1 gram of the recrystallized chemical (m.p.

<sup>1</sup> The experimental work described in this paper was carried out by Miss Joan Schechtman and Miss Eleanor E. Storrs.

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171°–173° C.) with 19 grams of pyrophilite in a mortar with a pestle. Commercial formulations of dichlone (2,3-dichloro-1,4-naphthoquinone) and Dyrene [2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine] containing 50 per cent active ingredient were used. The FDNB (1-fluoro-2,4-dinitrobenzene) was an experimental wettable powder containing 25 per cent active ingredient.

#### SOIL MIXTURES

The soil was a Gloucester loam top soil from the Yonkers, New York, area with a natural pH of 4.5 to 5. It was composted with manure and 5-10-5 fertilizer with sufficient limestone added to increase the pH to over 6, then it was stored in the field 2 to 3 years. Before use, it was screened to remove stones and other debris, and a portion of it was air dried. The moist soil had a pH of 6.4 and contained 17.5 per cent water; the air-dried soil had a pH of 6.2 and contained 1.6 per cent water. Each of the 4 fungicides was mixed at rates of 10 and 100  $\mu$ g. of active chemical per gram of soil with moist and air-dried soils. Mixing was done in a twin shell dry blender (The Patterson-Kelley Co. Inc., Stroudsburg, Penna.) for 30 minutes in batches of 1000 grams of soil. The soils were then placed in 2-quart jars, which were sealed with plastic screw top covers, and stored in a constant temperature room at  $26^{\circ} \pm 2^{\circ}$  C. Samples were withdrawn at various times for analysis.

#### ANALYTICAL PROCEDURES

*Captan.* Two-gram samples of soils treated with captan at the 100- $\mu$ g. rate were extracted with dichloromethane by the method previously described for 3,4-dichlorotetrahydrothiophene-1,1-dioxide (4). After decolorization with Norit-A mixture, fixative was added and the solvent evaporated. The residue was redissolved and divided into two equal portions and the active captan in each portion was determined by the pyridine-tetraethylammonium hydroxide procedure described earlier (3). The absorption maximum of the color produced is at 427  $m\mu$  rather than at 415  $m\mu$  as reported previously. Ten-gram soil samples were extracted when the application rate was 10  $\mu$ g.

*Dichlone.* Fifteen-gram samples of soils treated with dichlone at rates of 100  $\mu$ g. per gram were extracted with acetone for 20 minutes. The mixture was filtered through a fritted glass funnel and washed with four 5-ml. portions of acetone. The volume of the filtrate was made up to 50 ml. and a 35-ml. portion of it was treated with 10 ml. of 10 per cent dimethylamine reagent (1) in a 50-ml. volumetric flask. The volume was made up to 50 ml. with acetone and the absorbance of the resulting solution measured at 495  $m\mu$  against a blank prepared from soil that was not treated with dichlone. The solubility of dichlone in water and its stability in *M*/30 phosphate buffer were determined by the dichloromethane extraction procedure de-

scribed elsewhere (8) using the analytical technique given above (1). Since dichlone is photosensitive (2) these measurements were made in actinic glassware to avoid confounding hydrolysis and photolysis.

*FDNB*. Two-gram samples of soil treated at the 100- $\mu$ g. rate were extracted with 20 ml. of dichloromethane for 20 minutes as described previously. The mixtures were filtered and washed with four 5-ml. aliquots of solvent. The filtrate and washings were transferred to a 100-ml. volumetric flask and made up to volume with solvent. Five-ml. aliquots were evaporated to dryness in the presence of dimethoxytetraglycol as a fixative and the residue treated with 1 ml. of 70 per cent pyridine (v/v) and 5 ml. of water. After 20 minutes 1 ml. of 7*N* NaOH was added and the color was read at 548  $m\mu$  2 minutes later against a reagent blank (8). Proportionately larger aliquots of extracts from soils treated at rates of 10  $\mu$ g. per gram were used.

*Dyrene*. Decolorization with Norit-A mixture had to be avoided since the fungicide was sorbed by it. The extractions and analyses were made in the same manner as for FDNB except that absorbances were measured at 440  $m\mu$  against a soil blank (6).

#### PHYSICAL MEASUREMENTS

The hydrolysis rates of FDNB, Dyrene, dichlone, and captan in water containing 1 per cent acetone were measured by the dichloromethane extraction procedure described previously (8) using the analytical methods given above to detect residual chemical. The media contained *M*/30 phosphate buffer adjusted to pH 7.0, the initial concentration of fungicide was 5  $\mu$ g. per ml., and the temperature was maintained at 29° C. in a thermostatically controlled water bath. The experiments with dichlone were carried out in actinic glassware since this compound is known to be sensitive to light (2). The hydrolysis rate of captan was also determined in the absence of buffer.

The rate of disappearance of captan at an initial concentration of 20  $\mu$ g. per ml. was measured in the presence of 10,000 molecular equivalents of glycine under the conditions used for the hydrolysis studies. This procedure had to be modified as follows to study the disappearance of captan in the presence of glutathione because of the rapidity of the reaction. A solution containing 40.8  $\mu$ g. of glutathione per ml. in *M*/30 phosphate buffer at pH 7 was prepared under an atmosphere of nitrogen and the temperature adjusted to 29° C. Next 200  $\mu$ l. of a solution of captan in acetone at a concentration of 2000  $\mu$ g. per ml. were added to an Erlenmeyer flask. A 20-ml. aliquot of the glutathione solution was then added to the flask. The reaction was stopped at a pre-set time (measured with a stopwatch) by adding one ml. of 1*N* AgNO<sub>3</sub> to precipitate the glutathione and buffer. The suspension was then extracted with 20 ml. of dichloromethane,

and 5-ml. aliquots of the extracts were evaporated to dryness under a current of air and analyzed for residual captan. Time intervals between three and thirty seconds were used to obtain an estimate of the half time for the reaction.

The solubilities of these fungicides in water containing one per cent acetone were obtained by the technique described previously (7) except that an ultra-fine sintered glass funnel was used for filtration in place of Whatman No. 5 paper. In the case of captan it was not practical to hold the suspensions for 3 hours or centrifuge them because of its high rate of hydrolysis. Consequently the results reported on this compound are not exact. All solubility measurements refer to water containing 1 per cent acetone.

#### STABILITY IN MOIST AND DRY SOILS

The stability of Dyrene, FDNB, dichlone and captan in air-dried soil and in soil containing about 18 per cent moisture content was investigated by colorimetric methods which depend on the presence of reactive halogen atoms in the molecules. When these are lost either through hydrolysis or chemical reactions with organic compounds in the soil, the fungicides can no longer be detected (5). Hence it is possible to follow the deterioration of these compounds in soils by making measurements of the amount of chemically reactive material remaining at various time intervals. Of course this gives no direct evidence as to the actual fates of these fungicides in soil. They may hydrolyze, be attacked by microorganisms, undergo chemical reactions with metabolic debris or participate in interactions with components of the soil which are still unknown. Nevertheless fairly good estimates can be made of their over-all disappearance rates, and from these it is possible to speculate on the relative importance of the various pathways of attrition.

Dyrene was found to be the least stable of these compounds in soil under the conditions of these experiments. The time required for one-half of it to disappear when applied to moist soil at the 100- $\mu$ g. rate was only 0.5 day at 26° C. In air-dried soil, the half time was 2.5 days indicating that soil water accelerates breakdown, probably by supplying moisture films in which the compound can dissolve and react chemically. The breakdown of Dyrene followed first order (but not necessarily unimolecular) kinetics in both wet and dry soils, so that straight lines were obtained when the percentage of fungicide remaining was plotted in logarithmic units against time in days (Fig. 1).

During the first day, FDNB vanished from moist soil at about the same rate as Dyrene and its half time was about 0.5 day whether the initial application rate was 10 or 100  $\mu$ g. (Fig. 2). However, after this rapid initial drop, the rate of disappearance of the chemical slowed down and thereafter followed first order kinetics.

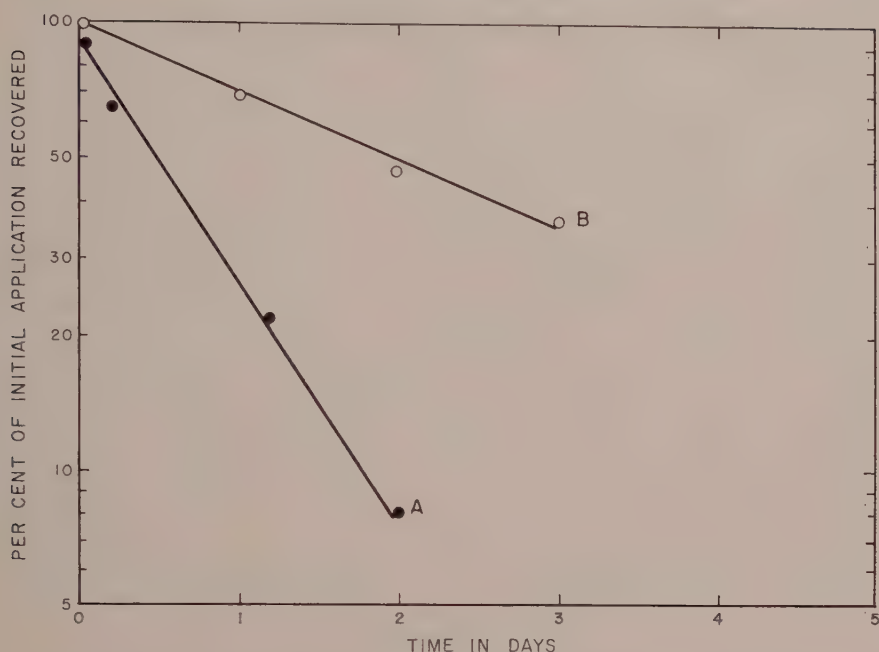


FIGURE 1. Recovery of Dyrene at various times at an initial application rate of 100  $\mu\text{g}$ . per gram from (A) soil containing 17.5 per cent moisture at pH 6.4 and (B) soil containing 1.6 per cent moisture at pH 6.2.

This rapid initial decrease occurred at both the 10- $\mu\text{g}$ . and 100- $\mu\text{g}$ . rates of application, so it does not seem likely that it was caused by the combination of FDNB with some highly reactive soil components present in very small amounts. If this were true, breakdown rate at the 100- $\mu\text{g}$ . application should slow down long before breakdown rate at the 10- $\mu\text{g}$ . application because of the more rapid exhaustion of the reactive soil component. It is evident from the data that this is not the case (Fig. 2). The reasons for this change in slope are as yet unknown. Nevertheless, because of this, FDNB has a longer over-all survival time in moist soil of the type used here than Dyrene, even though the half times are approximately the same. Thus at the end of 2 days more than twice as much FDNB was found in the soil, and at the end of 3 days the difference was 6- to 7-fold. The first order velocity coefficient for the disappearance of Dyrene from moist soil was 1.2 days<sup>-1</sup>. After this rapid initial loss, the disappearance of FDNB also followed first order kinetics, and the rate constant was 0.5 day<sup>-1</sup>. Thus after the first day, FDNB was about twice as persistent in moist soil as Dyrene. In air-dried soil FDNB was much more stable than Dyrene, the half time for its disappearance being greater than 50 days compared to 2.5 days for the latter compound. Even at the lower application rate of 10  $\mu\text{g}$ . of FDNB, 40 per cent of the chemical survived for 3



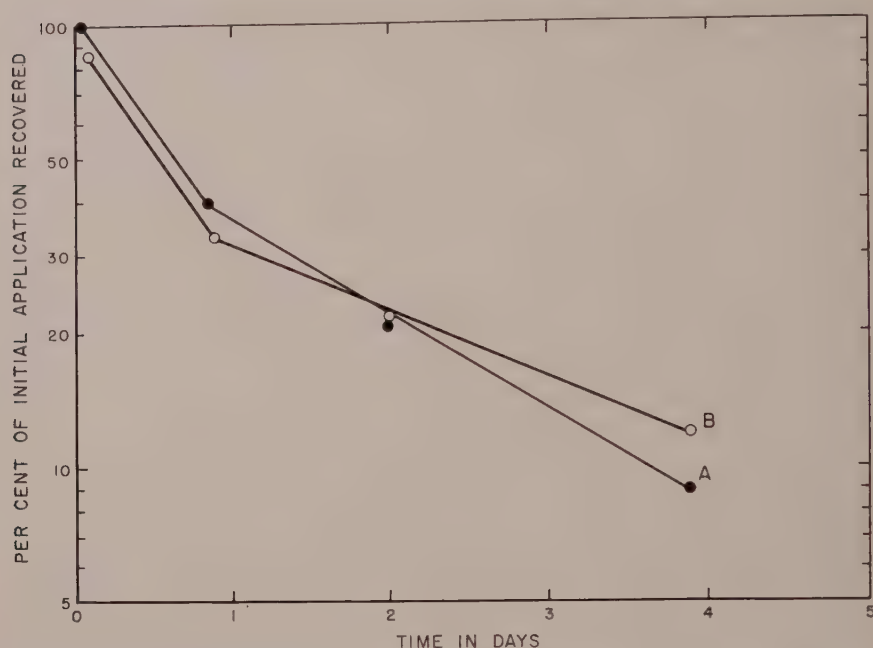


FIGURE 2. Recovery of FDNB at various times from moist soil at (A) an initial application rate of 100  $\mu\text{g.}$  per gram and (B) an initial application rate of 10  $\mu\text{g.}$  per gram.

months. The specific rate constant at the 100- $\mu\text{g.}$  level was found to be approximately  $0.01 \text{ day}^{-1}$  or about one-fiftieth of the value found in moist soil. The reasons for this enormous difference in the ratios of survival times of FDNB and Dyrene in moist and dry soils are unknown. It is unlikely that Dyrene is more mobile in a physical sense in dry soil than FDNB for their solubilities in water were found to be 10  $\mu\text{g.}$  per ml. and 400  $\mu\text{g.}$  per ml. respectively, and there is no reason to suspect that they would be sorbed in vastly different amounts by soil particles.

The breakdown of dichlone in moist soil, like that of Dyrene, followed first order kinetics. However, its disappearance rate was somewhat slower, the half time being about one day. At the end of 3 days only 12 per cent of the chemical remained at the initial application rate of 100  $\mu\text{g.}$  per gram of soil. However, in air-dried soil, breakdown was very much slower as shown by the finding that 45 per cent of the initial amount of chemical added to the soil was recovered at the end of 3 months. In this particular aspect of behavior it resembled FDNB rather than Dyrene.

In moist soil captan was the most stable compound of the 4 fungicides investigated. At application rates of 10 and 100  $\mu\text{g.}$  the half times were between 3 and 4 days. A rapid loss occurred during the first day (Fig. 3) but it was not so severe as found for FDNB. Thereafter first order kinetics

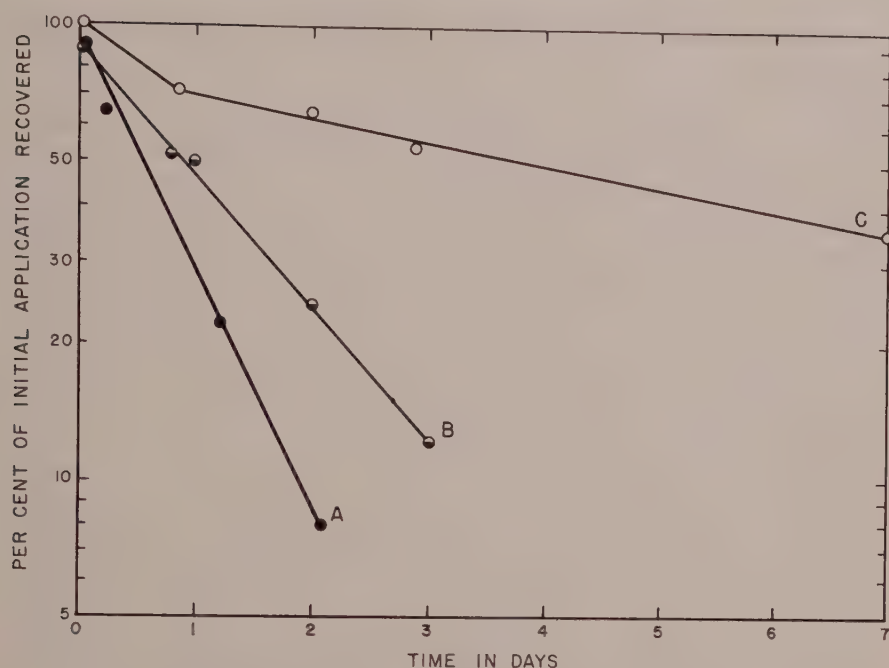


FIGURE 3. Stabilities of (A) Dyrene, (B) dichlone, and (C) captan in moist soil at initial application rates of 100  $\mu$ g. per gram.

were followed throughout the range in which measurements were made. The rate constant was about  $0.1 \text{ day}^{-1}$  so it can be seen (Table I) that captan was about 5 times more stable than FDNB, 7 times more stable than dichlone and about 12 times more stable than Dyrene, if the losses experienced during the first day are ignored. If half times only are considered, captan was about 3.5 times more stable than dichlone and 7 times

TABLE I

STABILITIES OF 4 ORGANIC FUNGICIDES IN MOIST AND DRY SOILS COMPARED TO THEIR HYDROLYSIS RATES IN pH 7 BUFFER AND WATER SOLUBILITIES. ALL SOIL APPLICATIONS WERE AT RATES OF 100  $\mu$ G. OF ACTIVE INGREDIENT PER GRAM OF SOIL

| Fungicide | Time in days for 50 per cent loss |               |                | $k$ in $\text{days}^{-1}$ (moist soil) | Water solubility, $\mu$ g. per ml. |
|-----------|-----------------------------------|---------------|----------------|----------------------------------------|------------------------------------|
|           | In dry soil                       | In moist soil | In pH 7 buffer |                                        |                                    |
| Dyrene    | 2.5                               | 0.5           | 20             | 1.2                                    | 10                                 |
| FDNB      | >50                               | 0.5           | 20             | 0.5*                                   | 400                                |
| Dichlone  | >50                               | 1             | 5              | 0.7                                    | 7                                  |
| Captan    | >50                               | 3.5           | 0.1            | 0.1*                                   | 8-70                               |

\* After 1 day.

more stable than FDNB and Dyrene under the soil conditions used in these tests.

As with FDNB, the reasons for the more rapid loss of captan during the first day are unknown. Earlier it was proposed that this might be caused by hydrolysis (3) but this now seems less likely since there is no obvious reason why hydrolysis rate should diminish with time in a buffered medium like soil. It was found that the hydrolysis rate of captan in unbuffered aqueous solution slowed down with time, while this was not true in  $M/30$

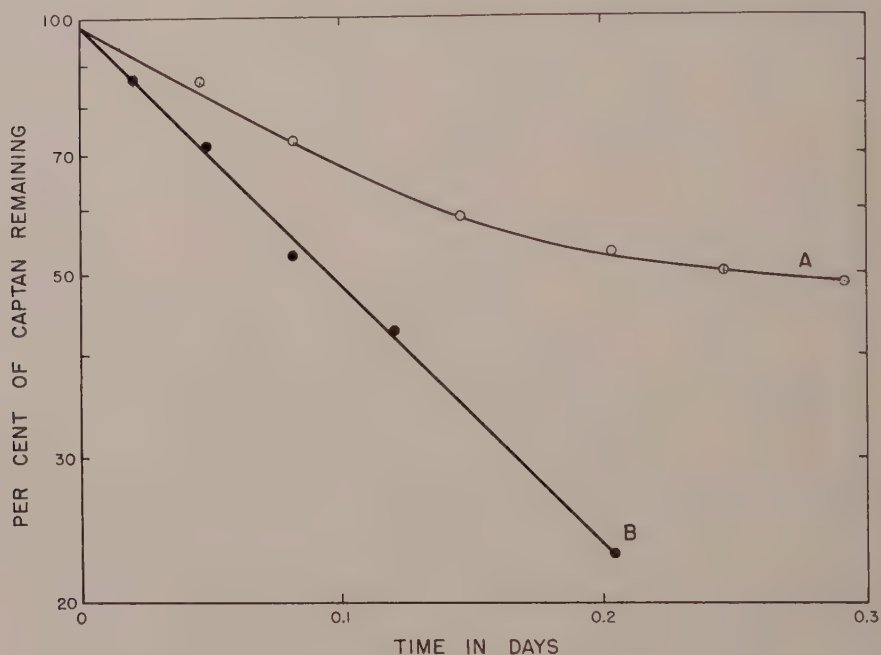


FIGURE 4. Stability of captan with time in (A) unbuffered solution and (B) in  $M/30$  phosphate buffer at pH 7.

phosphate buffer (Fig. 4). Thus the half time was increased from 0.1 day with buffer to 0.25 day without buffer. This suggests that one of the hydrolysis products inhibits the further breakdown of captan or an intermediate (9) in water but not in buffer. However, it would be surprising to find such an effect carry over to a medium such as soil.

Of course the possibility cannot be excluded that some of the anomalies observed in soil experiments arise from interactions with microorganisms and their metabolic products. Certainly it cannot be disputed that the soil flora has the potential of taking up and reacting with biocides of this type,

but whether the magnitudes of these effects would be great enough to be perceived in experiments of such short duration is unknown. Control experiments using sterilized soils might help to answer this question.

#### SOIL STABILITY AND HYDROLYSIS RATES

Several anomalies appeared in these data when attempts were made to compare stability in soil with hydrolysis rates in  $M/30$  phosphate buffer. FDNB and Dyrene were by far the most stable compounds in buffer solution with half lives of the order of 20 days. Dichlone had a half life of only 5 days in buffer when protected from light but it was about twice as stable as Dyrene in soil. More striking was the observation that captan had a half life of only 0.1 day in buffer compared with 3.5 days in soil. Thus while Dyrene was about 200 times more stable than captan in buffer, captan was 7 times more stable in soil than Dyrene on a half time basis, or about 12 times more stable if the slopes of the breakdown curves are used for comparison.

Differences in mobility in moisture films in the soil seem insufficient to account for this reversal in behavior. Mobility is probably a function of solubility, diffusion coefficient, and sorbability by soil particles, but of these factors, differences in solubility are likely to be the most important. The solubility of Dyrene in water containing 1 per cent acetone was found to be about 10  $\mu\text{g. per ml.}$  at 29° C. The solubility of captan in this medium could not be determined with certainty since it formed supersaturated solutions from which hydrolysis and slow precipitation occurred simultaneously. When 1 ml. of a concentrated solution of captan in acetone was added to 99 ml. of water, the filtrate that was obtained immediately contained 140  $\mu\text{g. of chemical per ml.}$  However, captan continued to come out of solution after filtration, and at the same time it hydrolyzed, so that at the end of 24 hours its concentration in equilibrium with the solid phase was 8  $\mu\text{g. per ml.}$  This latter result was probably not the true solubility of captan, but rather an equilibrium value dependent on its relative rates of solution and hydrolysis. Actually, the highest concentration of captan that could be prepared by the acetone dilution method without the rapid appearance of a solid phase was 70  $\mu\text{g. per ml.}$  Thus the true solubility of captan in water containing one per cent acetone is probably between 8 and 70  $\mu\text{g. per ml.}$  While this is not an accurately defined value it seems clear that the solubility of captan in aqueous media is about the same as or greater than that of Dyrene. Therefore, it is unlikely that the higher stability of captan in soil was caused by the fact that it did not dissolve in bound or free soil water as completely as Dyrene.

It is possible that the longer life of captan in soil arose in part from its higher degree of group specificity. This term was defined previously (8) as



the average reactivity of a toxicant with sulfhydryl groups divided by its average reactivity with amino groups.<sup>2</sup> For Dyrene this was found to be about

$$\text{G. S.} = \frac{k_{\text{RS}^-}}{k_{\text{RNH}_2}} = 10^2.$$

For FDNB it was found to be about  $10^3$ . An exact value has not been established for captan because of the inadequacy of present experimental methods, and uncertainty concerning the molecularity of its reactions.

The half time for the disappearance of captan in the presence of 2 molecular equivalents of glutathione in *M*/30 phosphate buffer at pH 7 was 15 seconds. This is close to the limits of error of the measurements. The half time in the presence of 10,000 molecular equivalents of glycine was 180 minutes after correction for the amount of chemical that disappeared through hydrolysis. If it is assumed that these reactions are bi-molecular, the ratio of the reactivity of captan with glutathione to its reactivity with glycine is seen to be of the order of  $10^6$ . Actually this assumption is not completely valid since the half times were not exactly proportional to concentration when the amounts of glycine were varied. Nevertheless, these values are sufficiently reliable to show that captan has a very much higher degree of group specificity than Dyrene, and in addition reacts considerably more slowly with compounds containing only amino groups (7, 8). For this reason it would not be as vulnerable as Dyrene to detoxication by amines in the soil, while the steady-state concentration of sulfhydryl groups in this medium might be too low to cause its rapid depletion (12).

This concept was introduced previously in an attempt to explain why FDNB is a more efficient soil fungicide than Dyrene, and it appears to have some measure of validity since it now has been shown that FDNB is somewhat more stable than Dyrene in moist soil and considerably more stable in dry soil. Furthermore, the higher water solubility of FDNB (400  $\mu\text{g. per ml.}$  vs. 10  $\mu\text{g. per ml.}$ ) should tend to offset in part its more highly specialized type of chemical reactivity. Captan in turn might be more stable in soil than FDNB because of its high group specificity and low solubility, despite its vulnerability to hydrolysis. It must be kept in mind, of course, that absolute levels of chemical reactivity as well as selective reactivity with certain chemical groups must be considered in discussing the longevity of chemicals in soils. For example, it is possible to synthesize

<sup>2</sup> This term could be applied to other ratios as well, for example the ratio of hydroxyl group reactivity to amino group reactivity, depending on the area of interest. It should be understood that this term applies only to reactions with small molecules and not to reactions with proteins.

s-triazines that are considerably less reactive than Dyrene but which have about the same degree of group specificity. It seems likely that such compounds would survive for longer periods in soils than Dyrene even though they are no more specialized in their chemical reactions.

Stability studies of the type described here have one common failing, whether they are based on highly specific methods of chemical analysis or more general bioassay techniques. The results are always dependent on the chemical, physical and microbiological properties of the soils used in the experiments, and these are difficult to define except in very general terms.

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# CHARACTERIZATION OF THE NONCARBONYL VOLATILES OF VANILLA BY GAS CHROMATOGRAPHY<sup>1</sup>

H. P. BURCHFIELD AND EDWARD A. PRILL

## SUMMARY

Vanilla extracts were treated with 2,4-dinitrophenylhydrazine to precipitate carbonyl compounds, and were then extracted with isopentane. After evaporation of the solvent, the residues were dissolved in 80  $\mu$ l. of acetone and 25  $\mu$ l. aliquots were chromatographed in gas chromatography equipment. Most authentic vanilla extracts yielded elution patterns with 4 characteristic peaks. These were designated as fractions *I*, *II*, *III* and *IV* in order of increasing retention time. Some samples yielded elution curves in which peak *III* was double, and in a few cases peak *II* was double. Extracts prepared from Tahiti beans gave patterns distinctly different from those obtained on Mexican and Madagascar beans. Increasing the pH of the extraction medium with  $\text{KHCO}_3$ ,  $\text{K}_2\text{CO}_3$  or KOH resulted in very large increases in the height of peak *II*. This method is useful for detecting adulterations in some commercial vanilla extracts which are not revealed by paper chromatography.

## INTRODUCTION

Paper chromatography has been shown to be very useful for establishing the authenticity of vanilla extracts and detecting adulteration with materials of botanical origin (2). However, flavoring materials may be used in formulating vanilla which cannot be found by this test because they do not fluoresce when exposed to ultraviolet radiation. Consequently, it seemed likely that gas chromatography might be a valuable supplement to paper chromatography for characterizing vanilla and establishing the presence of additives. Since vanillin is always present in such large amounts that it dominates the pattern, compounds containing carbonyl groups were removed prior to chromatography by precipitation with 2,4-dinitrophenylhydrazine. This should allow a much more meaningful analysis.

The results obtained on 21 samples of authentic vanilla extract and 3 commercial samples supplied as tenfold concentrates are described in this communication.

## MATERIALS AND METHODS

*Vanilla extracts.* Authentic vanilla extracts were prepared from beans of known origin and quality supplied by the Camax Co., Philadelphia, Penna. and the Thurston and Braidich Co., New York, N. Y. (Table I). Extractions were made using a menstruum of 73 ml. of 47.5 per cent alcohol for each 10 grams of beans with 25 per cent moisture. When the moisture content of the beans was greater or less than this figure, adjustments were made accordingly. The extracts were then diluted to 35 per cent alcohol

<sup>1</sup> This work was supported jointly by the Flavoring Extract Manufacturers' Association of the United States and the Vanilla Bean Association.

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TABLE I  
CLASSIFICATION OF AUTHENTIC VANILLA EXTRACTS ACCORDING TO PATTERN  
TYPE AND PREDOMINANT PEAK

| Code | Source of beans                                        | Pattern type | Predominant peak* |
|------|--------------------------------------------------------|--------------|-------------------|
| F-1  | Madagascar thirds                                      | A            | IV                |
| F-2  | Guadeloupes                                            | A            | IV                |
| F-5  | Comores thirds                                         | A            | IV(s)             |
| F-7  | Split Bourbon fourths (new)                            | A            | III(w)            |
| F-8  | Whole Bourbon thirds (new)                             | A            | IV                |
| F-9  | Whole Bourbon seconds (new)                            | A            | IV                |
| F-10 | Prime Bourbons (old)                                   | A            | IV                |
| F-14 | Mexican cuts (ordinary)                                | A            | III(s)            |
| F-16 | Madagascar fourths (splits)                            | A            | IV                |
| F-17 | Madagascar thirds (splits)                             | A            | IV(s)             |
| F-18 | Madagascar seconds (splits)                            | A            | III-IV            |
| F-3  | Mexican medianas                                       | B            | IV                |
| F-4  | Reunion seconds                                        | B            | IV                |
| F-11 | Whole Mexican buenas (old)                             | B            | IV                |
| F-12 | Whole Mexicans (superior, old)                         | B            | IV                |
| F-15 | Mexican cuts (good)                                    | B            | IIIa              |
| F-19 | Guadeloupe splits                                      | C            | III               |
| F-20 | Guadeloupe (nonsplits)                                 | C            | III               |
| F-21 | Vine ripened Mexican                                   | C            | III               |
| F-6  | Tahiti ( $\frac{1}{2}$ white and $\frac{1}{2}$ yellow) | T            | T-III(s)          |
| F-13 | Tahiti (white label)                                   | T            | T-III(s)          |

\* (s) Indicates that the peak is stronger than average and (w) that it is weaker.

to give a solution containing the extract of 10 grams of beans (25 per cent moisture basis) per 100 ml. The batch size was generally about 500 g. of beans. The extractions were made in a laboratory percolator of the type adopted as standard by the Flavoring Extract Manufacturers' Association (2). The duration of extraction was 36 hours and the temperature was held at 120° F. In one instance (M-1, Table II) the beans were re-extracted for two periods of 36 hours each with fresh charges of alcohol. The three extracts were then combined and the volume reduced to that of a singlefold<sup>2</sup> extract. Batch extractions were made in a 5-liter flask equipped with a mechanical stirrer and heated in a thermostatically controlled water bath. The ratio of beans to menstruum was the same as in the laboratory percolator. Extracts similar to those commonly believed to be marketed commercially (manufacturing type) were prepared by increasing the temperature, changing the time or adjusting the pH of the menstrua by adding 5*M* solutions of  $\text{KHCO}_3$ ,  $\text{K}_2\text{CO}_3$  or  $\text{KOH}$  (Table II). When bicarbonate or alkalis were used pH values were readjusted to 4.6 with *d-l* malic acid after extraction. All vanilla extracts were diluted with water to 35 per cent

<sup>2</sup> Single strength vanilla is defined as the solution obtained by extracting 10 grams of cured fruit of *Vanilla planifolia* (Andrews) with aqueous alcohol to make 100 ml. of extract. The water content of the beans is not specified. A vanilla tenfold is obtained by concentrating vanilla singlefold to ten times its normal strength.

TABLE II  
VARIATION IN HEIGHT OF PEAK II WITH EXTRACTION METHOD

| Code No. | Peak II height (cm.) | Adjuvant                               | pH range | Extraction method | Extraction time, hr. | Extraction temperature, ° F. |
|----------|----------------------|----------------------------------------|----------|-------------------|----------------------|------------------------------|
| M-3      | 3                    | None                                   | 4.6      | Batch             | 2                    | 173                          |
| M-2      | 4                    | None                                   | 4.6      | Batch             | 2                    | 120                          |
| M-1      | 5                    | None                                   | 4.6      | F.E.M.A.†         | 108                  | 110                          |
| M-12*    | 6                    | None                                   | 4.6      | Batch             | 2.5                  | 250                          |
| M-9      | 13                   | 9.6 g. KOH                             | 5.4-6.6  | F.E.M.A.          | 24                   | 160                          |
| M-7      | 13                   | 15 g. KHCO <sub>3</sub>                | 5.3-6.8  | Batch             | 2                    | 173                          |
| M-6      | 13                   | 21 g. K <sub>2</sub> CO <sub>3</sub>   | 6.2-6.5  | Batch             | 2                    | 173                          |
| M-4      | 13                   | 9.3 g. KOH                             | 6.1-6.6  | Batch             | 2                    | 173                          |
| M-8**    | 15                   | 9.3 g. KOH                             | 5.2-6.7  | Batch             | 2                    | 180                          |
| M-5      | 19                   | KOH                                    | 7.1-8.4  | Batch             | 2                    | 173                          |
| M-11     | > 20                 | 11 g. KHCO <sub>3</sub>                | 6.5-6.8  | F.E.M.A.          | 24                   | 160                          |
| M-10     | > 20                 | 21.5 g. K <sub>2</sub> CO <sub>3</sub> | 6.6-6.8  | F.E.M.A.          | 24                   | 160                          |

\* Autoclaved at 15 p.s.i.

\*\* Extracted with isopropanol and solvent removed.

† Three extractions of the same batch of beans.

alcohol content prior to analysis. The commercial tenfold vanilla samples designated as *Q*, *W* and *Y* were supplied by the Scientific Research Committee of the Flavoring Extract Manufacturers' Association.

*Synthetic flavors and plant extracts.* Prune juice concentrate, fluid extract of St. John's-bread, chicory concentrate, fluid extract of burdock root, fluid extract of kola and fluid extract of sarsaparilla were supplied by the Scientific Research Committee of the Flavoring Extract Manufacturers' Association. Solid extracts of guarana, fenugreek and horehound were purchased from S. B. Penick & Co., New York, N. Y. Five ml. of the liquid extracts or 5 g. of the solid extracts were used for the preparation of noncarbonyl fractions for gas chromatography. Palatone (3-hydroxy-2-methyl-4-pyrone) was obtained from the Dow Chemical Co., Midland, Mich., anisyl alcohol and coumarin from the Distillation Products Division of Eastman Kodak Co., and Imitation Vanilla Flavor 51.602A from Firmenich Inc., New York, N. Y.

*Reagents.* Dichloromethane and 2,4-dinitrophenylhydrazine were obtained from the Distillation Products Division of the Eastman Kodak Co. Phillips Petroleum Co. technical grade isopentane (b.p. 81° to 85° F.) was used for extraction. A test solution for carbonyl compounds was obtained by saturating 2*N* HCl with 2,4-dinitrophenylhydrazine. The evaporation residue from the vanilla extract was redissolved in Analytical Reagent grade acetone.

*Apparatus.* A liquid-liquid extractor of 100-ml. capacity obtained from the Scientific Glass Apparatus Co. (No. J-1631) was used. Although the capacity of the extractor is listed as 100 ml. it actually holds about 125 ml. Elution patterns were obtained on a Perkin-Elmer Model 154 Vapor Fractometer equipped with two Carbowax 1500 columns of the type described by Rhoades (3). Helium was used as the sweep gas at a pressure of 15 p.s.i. and an approximate flow rate of 100 ml. per minute. The operating temperature was 200° C. and the instrument was set at maximum sensitivity. It was necessary to clean the Fractometer with acetone after 10 to 20 runs.

*Procedure.* Place 100 ml. of singlefold vanilla extract containing 35 per cent alcohol or the equivalent of reconstituted tenfold in a 500-ml. Erlenmeyer flask and add 25 ml. of water, 3 ml. of 1:1 HCl, 0.8 g. of 2,4-dinitrophenylhydrazine and 4 ml. of dichloromethane. Close the neck of the flask with a piece of Saran wrap and shake on a rotary shaker for 4 hours. Test for completeness of removal of carbonyl compounds by filtering a small portion of the reaction mixture and adding 2,4-dinitrophenylhydrazine reagent to the filtrate. If the reaction is complete and no precipitate is formed add 2 ml. of dinitrophenylhydrazine reagent to the flask and shake it for an additional two hours with the cover removed to permit the dichloromethane to evaporate (Note A). Next transfer the suspension to a liquid-liquid extractor and extract for 24 hours at a rate of *ca.* 20 ml. per minute with isopentane (Note B) using an *extra* coarse disk. At the end of this time remove the flask containing the isopentane, seal the neck with Saran wrap and store at about 0° C. overnight (Note C). Filter the liquid through a fluted filter paper into a 250-ml. beaker and evaporate the solvent at about 40° C. under a current of clean dry air. Transfer the residue to a small vial by taking it up in several portions of dichloromethane. Evaporate the solvent under a current of air at 40° C. and redissolve the residue in 80  $\mu$ l. of A.R. grade acetone (Note D). Inject a 25- $\mu$ l. aliquot of this solution into a Perkin-Elmer Vapor Fractometer using a 50- $\mu$ l. syringe and obtain the elution pattern.

*Notes on procedure.* (A) The amount of dinitrophenylhydrazine used is enough to precipitate all the carbonyl-containing compounds present in authentic vanilla extract but more may be required if it is heavily fortified with vanillin. (B) Due to the low boiling point of the solvent it is necessary to use cold water in the condenser and also keep the temperature of the extraction tube below the boiling point. (C) Some solids separate out at this point; possibly dinitrophenylhydrazones of compounds other than vanillin since appreciable amounts of the latter hydrazone are not extracted under these conditions. (D) The residue from authentic vanilla (except Tahiti) will normally dissolve in this amount of solvent. However, some botanicals (kola and guarana, for example) and some commercial vanillas

yield rather large amounts of orange-yellow materials which will not dissolve in the specified amount of acetone. In these cases the supernatant was injected into the fractometer.

*Recording of data.* Results are recorded on a strip chart in terms of retention time in minutes plotted against an arbitrary scale of peak height at the maximum sensitivity setting of the instrument. For quantitative work it is necessary to determine the areas under the peaks, but for the present descriptive purposes peak height alone is used. The height of peak *II* (Table II) was measured as the distance in centimeters from the bottom of the trough on its descending side to the apex of the peak. Elution curves could be reproduced almost exactly with the same sample and instrument settings. However, differences in retention time of one to three minutes were sometimes observed on different samples run on different days.

## EXPERIMENTAL RESULTS AND DISCUSSION

### AUTHENTIC VANILLA EXTRACTS

The majority of the authentic vanilla extracts analyzed by gas chromatography gave elution patterns containing 4 principal regions (Fig. 1). For convenience these are referred to as peaks *I*, *II*, *III* and *IV* in order of increasing retention time. Peak *I* was often reduced to a shoulder on the descending arm of the solvent peak, and sometimes was absent altogether. This pattern, which was obtained on 11 of the 21 samples examined, was designated as type A. In five cases peak *III* was found to be double, so these vanillas were classified as type B. In three cases peak *II* was double, so these vanillas were called type C. No examples were found in which both *II* and *III* were double although it is not improbable that this could occur. All of the authentic vanilla samples examined could be classified under these three closely related categories with the exception of Tahiti. However, there was considerable variation within categories. In 12 instances peak *IV* was the predominant one, while in six cases *III* was the largest. In the pattern obtained on Madagascar second beans, the two peaks were about equal in height (Table I). Several extreme variations were found in the relative sizes of the peaks. Thus, in the pattern obtained on extract from Comores third beans the height of *IV* as measured from the trough between the two peaks was almost four times greater than *III*, while on the pattern of the extract from Mexican cuts the height of *III* was five times greater than *IV*.

There was no definite correlation between pattern type and quality ranking of the beans. The materials that were separated by the vapor fractometer were fragrant and undoubtedly contribute to aroma. Yet it was found that extracts of Comores thirds and Mexican cuts were richer in these components than the one prepared from the higher grade Mexican



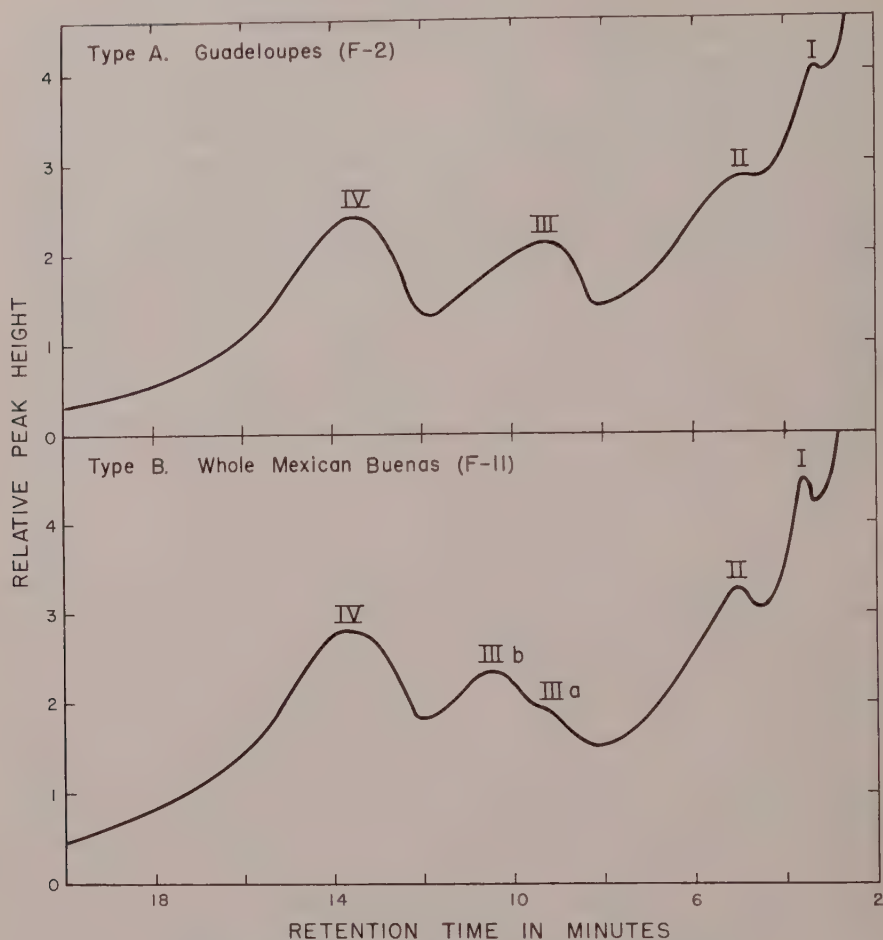


FIGURE 1. Elution patterns typical of extracts prepared from Mexican or Bourbon beans.

buenas. It may be of passing interest to note that 7 of the 8 samples belonging to types B and C were of New World origin while 9 of the 11 in type A were obtained from the Madagascar area.

Two extracts from authentic Tahiti beans were examined and in both cases the patterns were distinctly different from those described above (Fig. 2). In type A extracts the retention time was about 3.5 minutes for peak I, 4.5 to 5.5 minutes for peak II, 8 to 10 minutes for peak III and 11 to 14 minutes for peak IV with the equipment and instrument settings described in the materials and methods section of this paper. Three peaks were obtained on Tahiti with retention times of about 3.5 minutes (peak T-I), 6 to 8 minutes (peak T-II) and 8 to 10 minutes (peak

T-III). Peak T-I corresponded in position to *I* although it did not necessarily arise from the same compound. However, T-I was the highest peak in the Tahiti pattern, whereas *I* was often reduced to a shoulder on the solvent peak and was sometimes absent altogether in patterns obtained on Mexican and Bourbon type beans. T-II appeared to have a somewhat longer retention time than *II*, while T-III and *III* appeared at about the same places on the pattern, although once again this does not necessarily mean they arose from the same compounds. Peak *IV* was absent or greatly reduced in patterns obtained from Tahiti beans. Thus, Tahiti patterns were characterized by the predominance of *I*, a possible shift in *II*, and the virtual absence of *IV*. Since the Tahiti vanilla plant is a distinct botanical variety it is best to classify the peaks by a notation independent of that

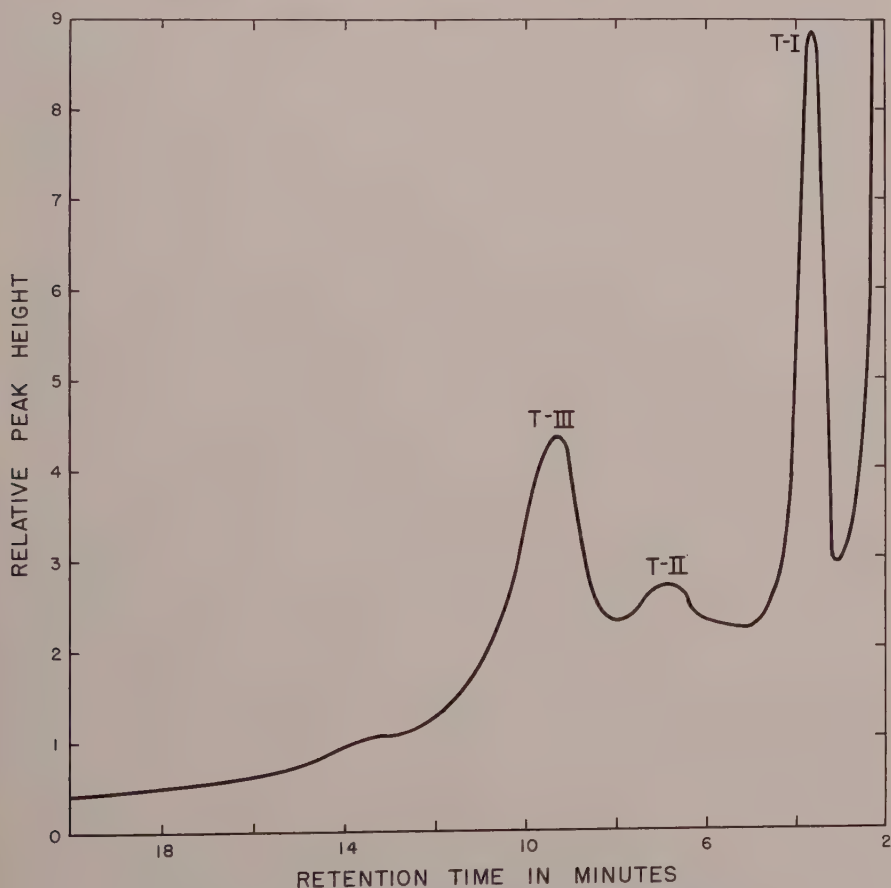


FIGURE 2. Elution pattern obtained on extract from Tahiti beans by gas chromatography.

used for Mexican and Madagascar beans until it is established whether they arise from the same chemical compounds.

#### INFLUENCE OF EXTRACTION METHODS

In addition to the possibilities of classifying vanilla beans according to noncarbonyl flavoring content and area of origin, this method may make it possible to detect the neutralization of natural acidity during extraction. When the extract from Bourbon third beans prepared by batch extraction at its natural pH of 4.6 was evaluated by this method, peak *I* was absent and peak *II* was about its average size. When the same beans were extracted in the presence of sufficient KOH to increase the pH to a maximum of 8.4, peak *II* became the predominant feature of the pattern (Fig. 3).

Evidently more of the material contributing to *II* was extracted at the higher pH, or more of it was formed from a precursor by hydrolysis. It was not necessary to increase the pH above 7 or to use alkali metal hydroxides to cause this. Thus, the addition of  $\text{KHCO}_3$  in increments to maintain the pH of the menstruum between 5.3 and 6.8 led to a similar increase in the height of *II* (Table II). This effect could be produced only by increasing the pH of the medium used for extraction, and did not result from increasing extraction time or temperature alone. Thus, the peak heights obtained on samples prepared by batch extraction for two hours and by extraction in the F.E.M.A. equipment for 108 hours with three charges of solvent were not significantly different (Table II). Autoclaving the beans at 15 p.s.i. steam pressure (250° F.) also failed to cause a significant increase in the height of *II*. However, in two cases out of three, prolonging the extraction in conjunction with increasing the pH increased the height of *II* to a greater extent than did increasing pH alone.

Since the retention time for peak *II* was not much longer than for peak *T-I* on Tahiti beans, a mixture of the noncarbonyl fractions derived from Tahiti and alkali-extracted Bourbon beans was made to see if it would be possible to distinguish between the effects of an increase in the pH of the menstruum and the replacement of part of the Bourbon beans with Tahitis (Fig. 4). As expected, the two peaks were cleanly resolved. Thus, any argument over whether a high peak at low retention time arose from the extraction method or the inclusion of some Tahiti beans could be settled by the addition of some authentic Tahiti extract to the suspected material as an internal standard.

#### USE OF ADDITIVES

Gas chromatography may sometimes be useful for detecting the presence of foreign botanicals in vanilla extracts, but in most cases paper chromatography should prove more valuable. Extracts from chicory, fenugreek and guarana were found to give characteristic patterns when ex-

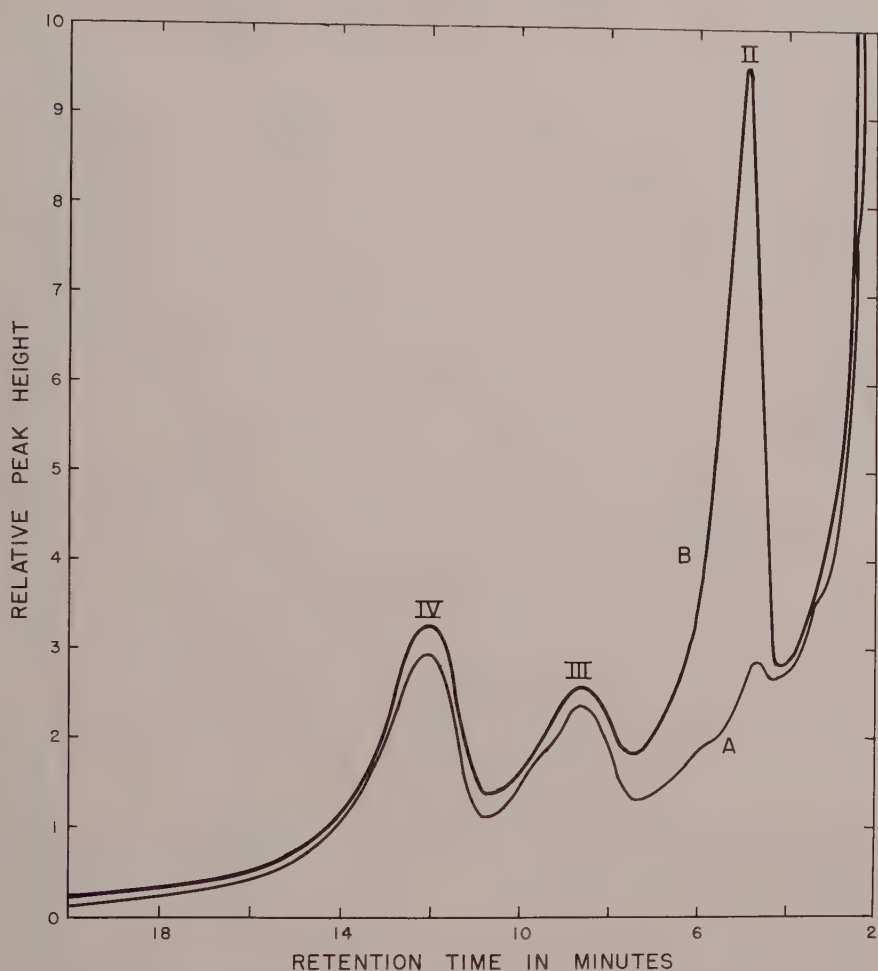


FIGURE 3. Effect of alkali used during extraction on the pattern obtained from Bourbon third beans. A. Beans extracted without alkali (M-2, Table II). B. Beans extracted in the presence of KOH (M-5, Table II).

amined by this procedure (Fig. 5). However, several other possible adulterants such as prune juice, St. John's-wort, St. John's-bread and burdock root gave weak patterns that would be very difficult to detect in the presence of vanilla.

The method may be more useful for the detection of flavoring materials which do not form insoluble 2,4-dinitrophenylhydrazones. For example, Firmenich's imitation vanilla flavor contained five components that could be resolved by gas chromatography under the conditions described. Four of these were removed by dinitrophenylhydrazine treatment, but a single



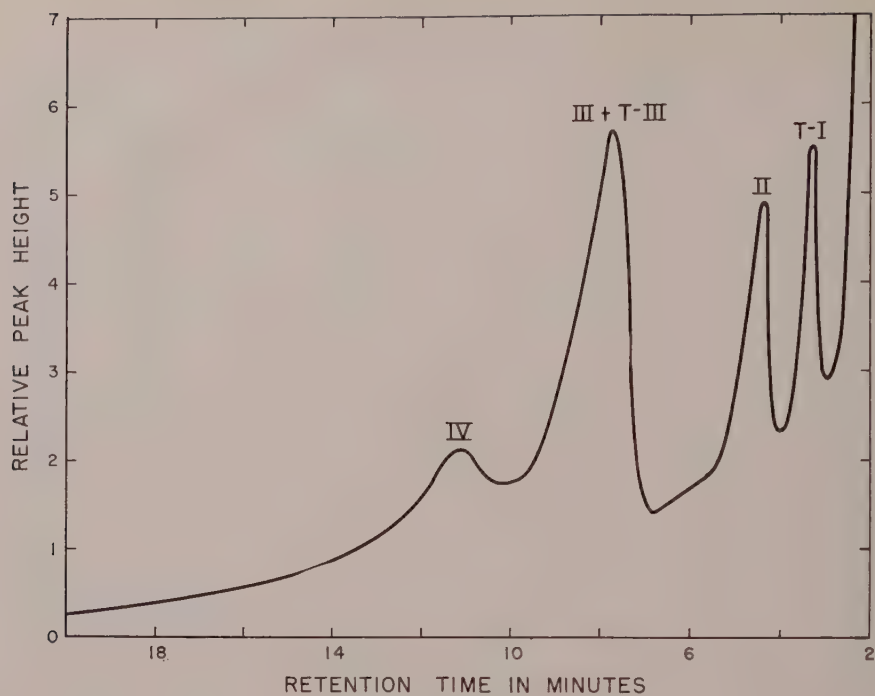


FIGURE 4. Pattern obtained on a mixture of the noncarbonyl fractions of Tahiti extract (F-6) and alkali-extracted Bourbon third beans showing the resolution of peaks T-I and II.

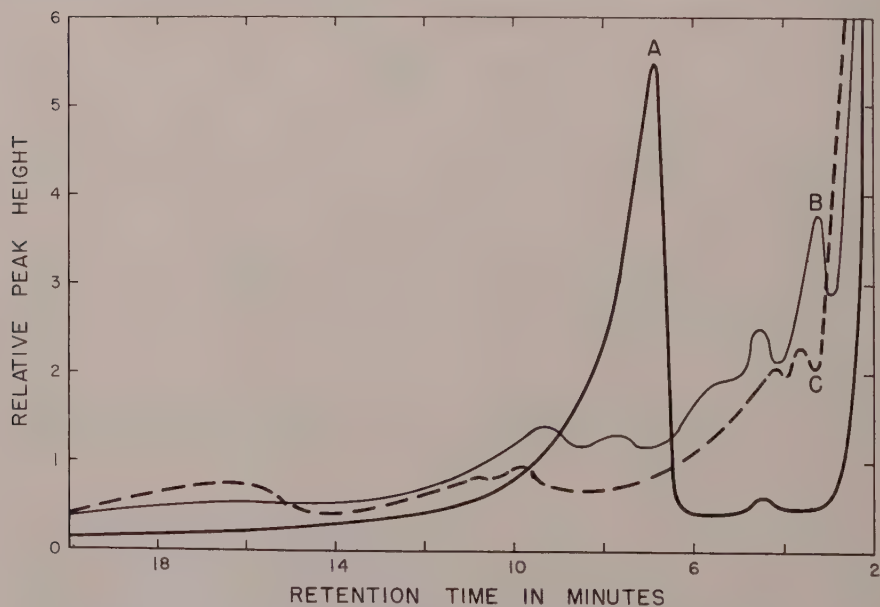


FIGURE 5. Patterns on noncarbonyl volatiles from other plant extracts. A. Fenugreek. B. Chicory. C. Guarana.

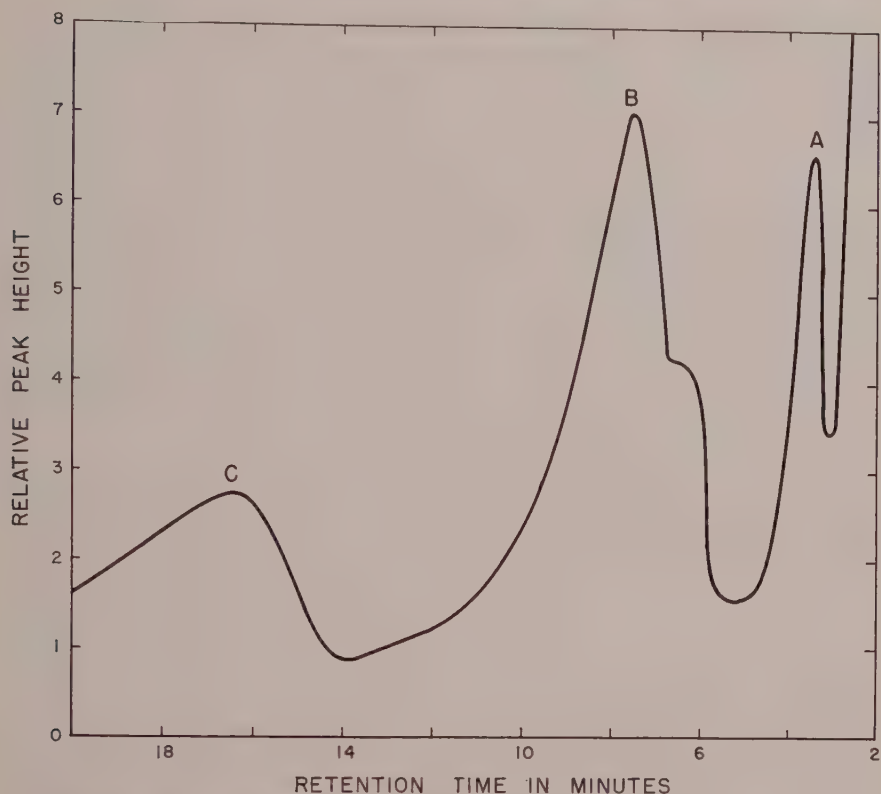


FIGURE 6. Elution pattern obtained on a mixture of potential vanilla flavoring adjuncts. A. Noncarbonyl fraction from Imitation Vanilla Flavor. B. Peak due to Palatone plus anisyl alcohol. C. Peak due to coumarin.

intense peak remained with the same retention time as peak T-I on Tahiti beans. It is interesting to note that this was the only component of Firmenich's flavor that survived the clean-up procedure. Peaks *II*, *III* and *IV* which probably represent some of the flavoring components of pure vanilla extracts prepared from Mexican and Madagascar beans were not found in this sample.

Other volatile materials which might be detected in this manner include Palatone, anisyl alcohol and coumarin (Fig. 6). The list of volatile materials which could be used to adulterate vanilla extract and which could be detected by this method is too long to permit anything more than a general survey of the possibilities.

Three commercial vanilla tenfolds were examined by this method and it appeared that one of them was grossly adulterated, a second contained little if any of the volatiles characteristic of authentic vanilla extract, while

the third might have been tampered with slightly. The pattern obtained on the sample coded tenfold *Y* was distinctly different and also was considerably more intense than those recorded on most authentic vanillas (Fig. 7), while the pattern obtained on *Q* was not much more intense than would be found on a 2,4-dinitrophenylhydrazine blank. The small peak found on *Q* at a retention time of about 4.5 minutes is characteristic of extracts from many plant sources including St. John's-wort, St. John's-bread, etc. Tenfold *W* appeared to be based on authentic vanilla beans of type B used in reasonable quantity. Peak *II* was not high enough to suggest the use of alkali during the extraction, but peak *I* was abnormally high for extracts based on Mexican or Bourbon vanilla beans. This suggests that some Tahiti beans may have been included, or an artificial flavoring material with the same retention time as one of the components of Firmenich's Imitation Vanilla Flavor may have been used.

Tenfold *W* gave a fluorescent pattern equivalent to authentic although somewhat dilute vanilla extract when examined by paper chromatography. On the other hand, both tenfolds *Y* and *Q* showed some evidence of adul-

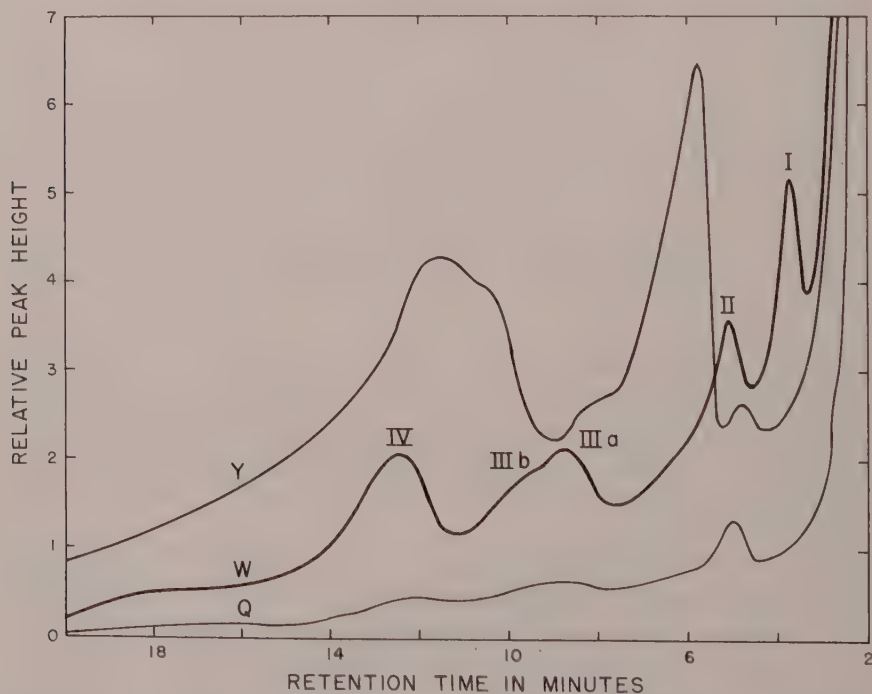


FIGURE 7. Patterns obtained on commercial tenfolds *Y*, *W* and *Q*. Note that tenfold *W* gives a type B pattern with peak *I* abnormally high while the patterns of tenfolds *Y* and *Q* do not resemble those of any of the authentic extracts examined in this investigation.

teration, although this was not strong. The fact that these materials were so easily shown to be spurious by gas chromatography suggests that this method will be a valuable adjunct to the paper chromatographic method described previously (1).

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# THE PROPAGATION OF LARIX FROM SOFT-WOOD CUTTINGS

CLYDE CHANDLER

## SUMMARY

Cuttings used in the propagation experiments were from trees of *Larix decidua* Mill., *L. leptolepis* Gord., *L. laricina* Koch, *L. Gmelinii* Litvinov, *L. eurolepis* Henry (hybrid) and from unnamed hybrid seedlings produced at Boyce Thompson Institute.

From 1948 to 1956 various experiments were made in an attempt to find a suitable method for rooting soft-wood cuttings of larch. Only occasional rooting took place in a conventional greenhouse bench or in a Wardian case. Sugars and fungicide treatments proved ineffective. Success was attained in 1956, 1957 and 1958 tests by the use of a Watco mist control unit installed in the greenhouse. Sand was used as a rooting medium. Two- to 4-year-old trees furnished best cutting material and least rooting was obtained from cuttings from 27- to 30-year-old stock. Treatment with Hormodin 3 promoted rooting of cuttings from all trees above 4 years of age. Cuttings taken in August and September proved superior to those taken in May or June. Tip cuttings from terminal shoots rooted more easily than the basal portions.

These findings now make it possible to secure enough cutting material for clonal field tests.

## INTRODUCTION

Any forest tree improvement program, whether it be approached through provenance testing or through plant breeding, has as its aim the selection of fast-growing, disease-resistant and well-formed individuals which are designated as "elite" trees. Before these elite trees can be evaluated it is necessary to obtain enough clonal propagations to place an adequately replicated experimental planting in a field test where each clone may be compared with all the others. Such clones may be established by grafting or by soft-wood cuttings. Clonal material of larch is usually established by grafting (5, pp. 181-182; 6, p. 395). However, if grafts are used the question of the influence of root-stock upon the scion material is ever present. It, therefore, seems logical to propagate the elite trees through cuttings.

Hyun (1) rooted cuttings from 10-year-old *Larix Kaempferi* by soaking the cuttings in a solution containing indoleacetic acid (IA), indolebutyric acid (IB),  $\alpha$ -naphthaleneacetic acid (NA) and vitamin B<sub>1</sub> for 24 hours. He found that thirty cuttings treated on March 23 gave 6.3 per cent rooted cuttings. On March 26 twenty-five cuttings were soaked in each of the following solutions: (a) IA 40 p.p.m., IB 40 p.p.m., vitamin B<sub>1</sub> 10 p.p.m., vitamin B<sub>6</sub> 10 p.p.m., nicotinic acid (N) 10 p.p.m., glucose (G) 10 p.p.m., sucrose 2.5 per cent; (b) NA 70 p.p.m., vitamin B<sub>1</sub> 10 p.p.m., vita-

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min B<sub>6</sub> 10 p.p.m., N 10 p.p.m., G 10 p.p.m., sucrose 2.5 per cent; (c) vitamin B<sub>1</sub> 10 p.p.m., vitamin B<sub>6</sub> 10 p.p.m., N 10 p.p.m., G 10 p.p.m., sucrose 2.5 per cent. Sixteen, 12 and 8 per cent of rooting, respectively, were obtained on September 25 from these treatments. Sixty cuttings collected on March 17 and treated with IA 20 p.p.m., IB 20 p.p.m., vitamin B<sub>1</sub> 10 p.p.m., and 5 per cent sucrose failed to root.

Kurdiani (4) rooted European larch in 1908 by placing soft-wood cuttings in sand in a cold frame. Frames were covered with glass sashes painted with lime. They were shaded from 10 A.M. to 4 P.M. and were covered at night with raffia mats. The cuttings were watered three times daily. After two or three months 56 to 75 per cent rooting was obtained. He concluded that cuttings of *Larix europaea* rooted more easily than pine cuttings.

Komissarov (3) applied growth substances to *Larix sibirica* cuttings. By soaking cuttings from 3- and 10-year-old trees in 0.005 to 0.010 per cent  $\beta$ -indolylacetic acid for 24 and 48 hours 85 and 70 per cent rooting was obtained, while for untreated cuttings of the same age 20 to 30 per cent rooting was obtained from 3-year-old trees and only 10 per cent for 10-year-old trees.

Komissarov in an earlier paper (2) reported a difference in the rooting of cuttings taken in June (26 per cent) which he described as nonwoody and those taken in July (6 per cent) which he described as woody. Cuttings soaked in water gave no rooting.

Larsen (5, p. 182) states that "By using soft wood cuttings in the summer, quite a lively root-formation can be obtained, but the difficulty lies in getting good shoot formation and handling the transplantation so that it has not come into general use. Nevertheless good plants can be raised in that way and efforts to devise a satisfactory technique ought to be continued. Propagation by cuttings can present considerable advantages."

The larch improvement program at Boyce Thompson Institute for Plant Research in Yonkers, New York was begun in 1948. About 100 trees of four different species were growing in the Arboretum. The individual trees within a species showed marked differences in size, form and degree of flowering. It was desirable to propagate the slow-growing trees for a comparison with fast-growing ones to determine if this difference in rate of growth is genetically controlled.

#### ROOTING WITHOUT MIST

From 1948 to 1956 various experiments were made in an attempt to find a suitable method for rooting soft-wood cuttings. Results and information were not consistent. From thousands of cuttings only occasional roots were formed. The regular greenhouse bench as well as a Wardian case in the greenhouse or in the field was tried without success. Arasan (tetramethylthiuram disulfide) and Spergon (tetrachloro-*p*-benzoquinone)

in the medium did not prevent rotting. In these experiments, mannitol, sucrose, lactose, and dextrose were ineffective. Cuttings in a cold frame sealed with polyethylene and provided with partial shade failed to root.

### ROOTING WITH MIST

#### MATERIALS AND METHODS

In August, 1956 a Watco mist control unit supplied by the Water Cooling Corporation of New York was installed in the greenhouse bench. This was set to provide six seconds of mist every six minutes. Cuttings were placed in sharp builder's sand, firmed and thoroughly watered. No further watering was necessary. An inch of coarse gravel underneath the sand provided drainage. The greenhouse glass was painted for shade. Cutting types included soft wood from terminal and lateral growth and were from four to six inches in length. In summer when growth was extended, two cuttings were made from each terminal and each lateral shoot. The basal portions are designated as terminals and laterals and tips from terminal and lateral cuttings are called terminal and lateral tips throughout this report. Hormodin powders 1, 2, and 3 obtained from Merck & Co., Inc., Rahway, New Jersey were applied uniformly as dusts to the basal ends of cuttings at the time of planting.

Cuttings were obtained from species and hybrids of different ages which include *Larix decidua* Mill., *L. leptolepis* Gord., *L. laricina* Koch, *L. Gmelinii* Litvinov, *L. eurolepis* Henry (hybrid) and over a hundred hybrid seedlings produced at Boyce Thompson Institute.

#### RESULTS

1956. In 1956 a total of 3,161 terminal cuttings were taken from five classes of larch which included *L. decidua*, *L. leptolepis*, *L. eurolepis* (hybrid) and *L. Gmelinii*. These trees were eight years old from date of seed planting. Cuttings were made in August and September and were placed in three types of media which included sand, one-half sand and one-half peat, and a mixture of sand, peat and sphagnum in equal parts. Some were treated with Hormodin 3 and others were not treated. Of these cuttings 261 (8 per cent) rooted. These data indicate that cuttings of *L. leptolepis* rooted somewhat more frequently than those from the other classes. Cuttings taken in August gave better rooting than those taken in September. Cuttings in sand and the mixture of sand, peat and sphagnum rooted better than those in sand and peat. Hormodin 3 stimulated rooting.

The 455 cuttings from trees 29 years of age gave only 1 per cent rooting while the 497 from younger trees (6 to 7 years old) gave 12 per cent rooting.

Mallet cuttings showed poor rooting. On the other hand, 12 to 21 per cent of the tips of terminal and lateral cuttings formed roots.

1957. In 1957 a total of 9,563 cuttings were placed under mist and



TABLE I  
EFFECT OF AGE OF LARIX TREES UPON THE ROOTING OF SOFT-WOOD CUTTINGS  
(1957 TESTS)

| Age of trees,<br>years | No. of<br>cuttings | No.<br>rooted | Per cent<br>rooted |
|------------------------|--------------------|---------------|--------------------|
| 27 to 30               | 814                | 54            | 7                  |
| 9                      | 7226               | 724           | 10                 |
| 8                      | 381                | 54            | 14                 |
| 7                      | 380                | 40            | 11                 |
| 4                      | 538                | 171           | 32                 |
| 3                      | 166                | 59            | 36                 |
| 2                      | 58                 | 22            | 38                 |

1,124 rooted (12 per cent). These cuttings were from trees of different ages as well as of various species and hybrids. Cuttings were of different types, received different treatments and were taken during May, June, August and September. Rootability of cuttings depends on all of these factors and probably many more which have not been investigated up to the present time.

Age is a very definite factor which influences the rooting of soft-wood cuttings. The highest percentages of rooting were obtained from 2- to 4-year-old seedlings and the least rooting was with cuttings from 27- to 30-year-old trees as shown in Table I. Among the 8-year-old trees one *L. leptolepis* seedling gave excellent rooting.

Of the 380 cuttings from thirteen 7-year-old trees only 40 rooted (11 per cent). However, if we consider only those treated with Hormodin 3, cuttings from 7 of the 13 trees gave above 28 per cent rooting with a maximum percentage of 75.

Since the largest number of cuttings in any one age group was 7,226 from 9-year-old seedlings, these data are summarized in Table II. These were from 32 different trees which represent four species and one hybrid. Terminal cuttings of *L. decidua* gave no rooting. There was no significant difference in the rootability of cuttings from trees of the other species or the hybrid, *L. eurolepis*. However, individual trees within a species varied from 1 to 20 per cent for *L. leptolepis* and from 1 to 22 per cent for the hybrid *L. eurolepis*.

TABLE II  
ROOTING OF CUTTINGS FROM DIFFERENT CLASSES OF 9-YEAR-OLD LARIX TREES

| Class of<br><i>Larix</i> | No. of<br>trees | No. of cuttings |         |       | Per cent rooted |         |       |
|--------------------------|-----------------|-----------------|---------|-------|-----------------|---------|-------|
|                          |                 | Terminal        | Lateral | Total | Terminal        | Lateral | Total |
| <i>L. decidua</i>        | 5               | 230             | —       | 230   | 0               | —       | 0     |
| <i>L. leptolepis</i>     | 16              | 1868            | 2447    | 4315  | 10              | 12      | 11    |
| <i>L. eurolepis</i>      | 9               | 877             | 1636    | 2513  | 10              | 9       | 9     |
| <i>L. Gmelinii</i>       | 1               | 56              | —       | 56    | 11              | —       | 11    |
| <i>L. laricina</i>       | 1               | 53              | 59      | 112   | 11              | 12      | 12    |

TABLE III  
ROOTING OF CUTTINGS TAKEN FROM LARIX TREES AT DIFFERENT  
TIMES DURING THE GROWING SEASON

| Month of cutting | No. of trees | No. of cuttings |         |       | Per cent rooted |         |       |
|------------------|--------------|-----------------|---------|-------|-----------------|---------|-------|
|                  |              | Terminal        | Lateral | Total | Terminal        | Lateral | Total |
| May              | 26           | 523             | 1018    | 1541  | 10              | 11      | 11    |
| June             | 32           | 1400            | 1688    | 3088  | 6               | 7       | 6     |
| August           | 25           | 1158            | 937     | 2095  | 18              | 17      | 18    |
| September        | 30           | 1611            | 1228    | 2839  | 16              | 11      | 14    |

These cuttings were also taken during four different months including May, June, August and September as shown in Table III. Cuttings taken in May gave better rooting than those taken in June. The higher percentages of rooting obtained in August and September were due in part to the material, some of which was from younger trees. The higher percentages of rooted cuttings for individual trees may be seen in Table IV where data from the five trees from which best rooting was obtained during each of the four months are tabulated. Sixteen different trees supplied material for these tests. With the exception of three (numbers 4, 5 and 16) these were either *L. leptolepis* or the hybrid, *L. eurolepis*. Cuttings from *L. decidua*, *L. Gmelinii* or *L. laricina* were never among the top five lots in rooting ability. Four trees (numbers 3, 8, 9 and 10) rated among the five best sources of cuttings for two different months.

Since earlier experiments indicated that Hormodin stimulated better root development some of these cuttings were treated with Hormodin powders 1, 2 and 3 before planting while others were not treated and served as controls. These data substantiate the earlier findings as shown in Table V. Cuttings from all seedlings 3 to 30 years of age showed stimulated rooting with Hormodin treatment. The rooting of cuttings of more mature wood taken later in the season (August and September) from 9-year-old trees was increased from 8 to 31 per cent by the use of Hormodin 3. On the other hand, cuttings from very young seedlings (2 years old) rooted equally well with or without hormone treatment.

Hormodin was effective on cuttings from eight different 3- and 4-year-old hybrid trees which gave an average of 33 per cent rooting, though the individual trees varied from 12 to 60 per cent. Tips from terminal cuttings rooted better than the basal portion of the cuttings (45 and 21 per cent, respectively). The highest percentage of rooting for terminals of any individual plant was 40 while 83 per cent of terminal tips rooted. Again, in hybrids as in species noted above, cuttings from 2-year-old trees did not require Hormodin for rooting.

1958. In the 1958 tests rooting up to 13 per cent was obtained in *L. decidua* cuttings while all cuttings taken from this species in 1957 failed

TABLE IV

HIGHEST PERCENTAGES OF ROOTING OBTAINED FROM CUTTINGS TAKEN FROM FIVE TREES AT EACH OF FOUR DIFFERENT MONTHS DURING THE GROWING SEASON

| Month of cutting | Class of <i>Larix</i>  | Tree No. | No. of cuttings |      |       | Per cent rooted |      |       |
|------------------|------------------------|----------|-----------------|------|-------|-----------------|------|-------|
|                  |                        |          | Term.           | Lat. | Total | Term.           | Lat. | Total |
| May              | <i>L. leptolepis</i> * | 1        | 18              | 32   | 50    | 50              | 66   | 58    |
|                  | <i>L. leptolepis</i>   | 2        | 15              | 20   | 34    | 53              | 20   | 34    |
|                  | <i>L. leptolepis</i>   | 3        | —               | 86   | 86    | —               | 20   | 20    |
|                  | Hybrid**               | 4        | 20              | 40   | 60    | 5               | 25   | 18    |
|                  | Hybrid†                | 5        | 21              | —    | 21    | 33              | —    | —     |
|                  |                        |          | 74              | 178  | 252   | 34              | 34   | 30    |
| June             | <i>L. leptolepis</i>   | 6        | 68              | 38   | 106   | 13              | 29   | 19    |
|                  | <i>L. leptolepis</i>   | 7        | 21              | 106  | 127   | 24              | 12   | 14    |
|                  | <i>L. eurolepis</i>    | 8        | 37              | 59   | 96    | 16              | 12   | 14    |
|                  | <i>L. eurolepis</i>    | 9        | 26              | —    | 26    | 39              | —    | 39    |
|                  | <i>L. eurolepis</i>    | 10       | 22              | —    | 22    | 32              | —    | 32    |
|                  |                        |          | 174             | 203  | 377   | 21              | 15   | 18    |
| August           | <i>L. leptolepis</i>   | 11       | 48              | 14   | 62    | 54              | 14   | 45    |
|                  | <i>L. leptolepis</i>   | 12       | 72              | 70   | 142   | 28              | 30   | 29    |
|                  | <i>L. leptolepis</i>   | 3        | 98              | 29   | 127   | 13              | 52   | 22    |
|                  | <i>L. eurolepis</i>    | 13       | 74              | 142  | 216   | 34              | 25   | 28    |
|                  | <i>L. eurolepis</i>    | 10       | 20              | —    | 20    | 65              | —    | 65    |
|                  |                        |          | 312             | 255  | 567   | 31              | 29   | 30    |
| September        | <i>L. leptolepis</i>   | 14       | 88              | 124  | 212   | 7               | 34   | 23    |
|                  | <i>L. eurolepis</i>    | 8        | 59              | 118  | 177   | 41              | 25   | 31    |
|                  | <i>L. eurolepis</i>    | 9        | 44              | —    | 44    | 50              | —    | 50    |
|                  | <i>L. eurolepis</i>    | 15       | 60              | —    | 60    | 60              | —    | 60    |
|                  | Hybrid††               | 16       | 20              | —    | 20    | 70              | —    | 70    |
|                  |                        |          | 271             | 242  | 513   | 38              | 30   | 34    |

\* *L. leptolepis* × *L. leptolepis*.

\*\* *L. decidua* × *L. leptolepis* β *murrayana* Maxim.

† *L. eurolepis* × *L. leptolepis* β *murrayana* Maxim.

†† (*L. leptolepis* × *L. occidentalis*) × *L. leptolepis*.

TABLE V

EFFECT OF HORMODIN POWDERS ON ROOTING OF CUTTINGS FROM LARIX TREES OF DIFFERENT AGES

| Age, years | No. of cuttings treated* |                |                |                | % Of cuttings rooted |                |                |                |
|------------|--------------------------|----------------|----------------|----------------|----------------------|----------------|----------------|----------------|
|            | Ck.                      | H <sub>1</sub> | H <sub>2</sub> | H <sub>3</sub> | Ck.                  | H <sub>1</sub> | H <sub>2</sub> | H <sub>3</sub> |
| 27 to 30   | 215                      | 198            | 203            | 108            | 2                    | 3              | 11             | 11             |
| 9          | 2828                     | 793            | 814            | 2791           | 5                    | 4              | 6              | 18             |
| 7 to 8     | 273                      | 91             | 99             | 298            | 3                    | 14             | 16             | 20             |
| 3 to 4     | 347                      | 10             | 10             | 337            | 28                   | 50             | 20             | 38             |
| 2          | 29                       | —              | —              | 29             | 38                   | —              | —              | 38             |

\* Cuttings not treated (Ck.) or treated with Hormodin powders 1, 2 or 3 (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>).

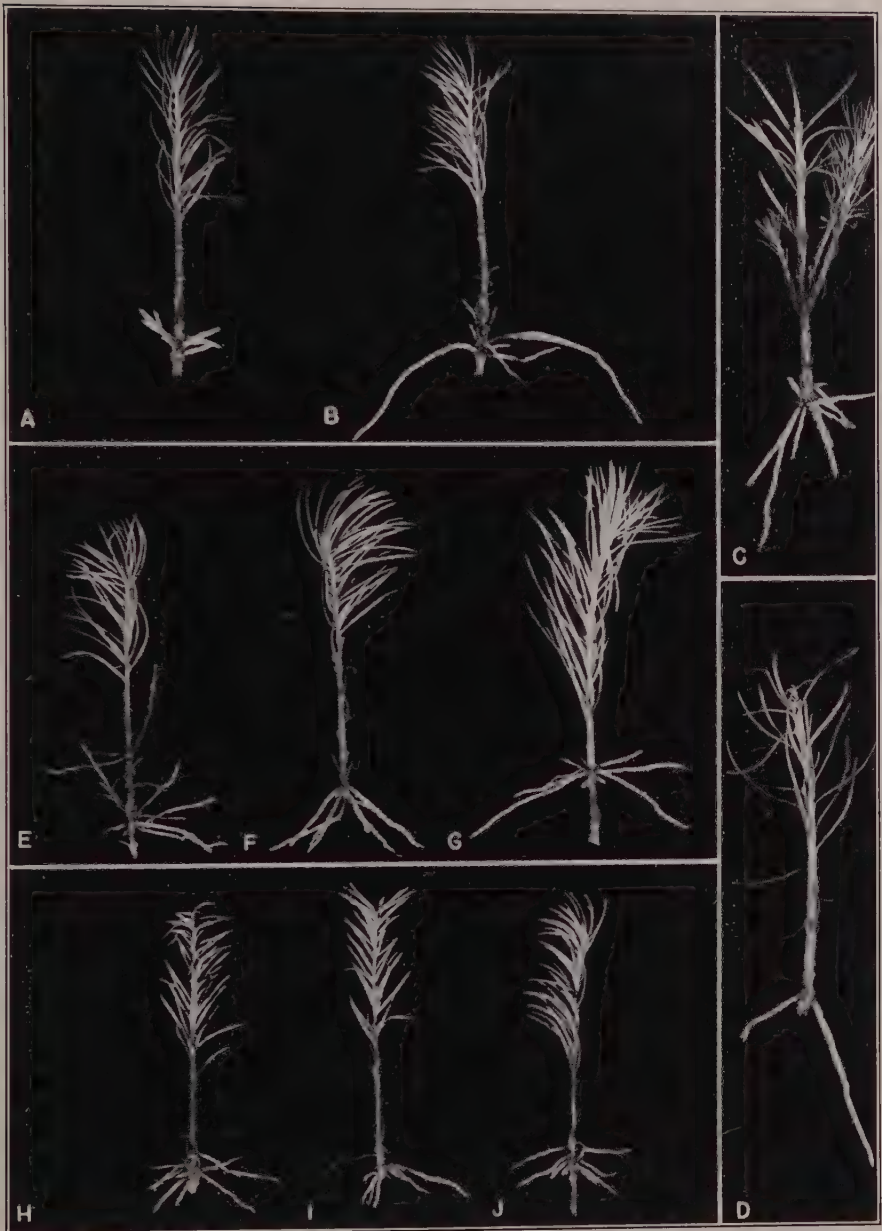


FIGURE 1. *Larix* cuttings rooted with mist. A, B, tips of terminal cuttings and C, D, mid-section immediately below tip from *Larix leptolepis* 9 years of age. E, F, G, terminal tips and H, I, J, lateral tips from hybrid (*L. leptolepis*  $\times$  *murrayana*  $\times$  *L. eurolepis*) 4 and 5 years of age, respectively ( $\times 0.28$ ).



TABLE VI  
EFFECT OF AGE OF LARIX TREES UPON THE ROOTING OF SOFT-WOOD CUTTINGS  
(1958 TESTS)

| Age of trees,<br>years | No. of<br>trees | No. of<br>cuttings | Per cent rooted |     |      | No. of<br>trees above<br>average |
|------------------------|-----------------|--------------------|-----------------|-----|------|----------------------------------|
|                        |                 |                    | Average         | Low | High |                                  |
| 28 to 31               | 5               | 80                 | 24              | 4   | 53   | 2                                |
| 10                     | 27              | 2287               | 23              | 5   | 46   | 13                               |
| 8 to 9                 | 31              | 1155               | 22              | 3   | 54   | 15                               |
| 4 to 5                 | 30              | 1165               | 35              | 3   | 95   | 12                               |
| 3                      | 12              | 180                | 35              | 5   | 90   | 5                                |
| 2                      | 3               | 60                 | 17              | 5   | 30   | 1                                |

to root. In general, however, results of the 1958 trials were in agreement with those of the previous year.

In 1958 a total of 13,502 cuttings were placed under mist to obtain material for clonal field tests. The methods were the same as those employed in 1957. Rooting was obtained from 108 different trees as shown in Table VI. Eleven of these trees gave from 75 to 100 per cent rooting while 33 others gave above 50 per cent rooting. Rooting was obtained on various types of cuttings as shown in Figure 1, A to J. Summaries of these data for 1958 substantiate the following facts as shown by previous data: cuttings from trees of different species varied in their ability to root; individual trees within the species also varied in the number of rooted cuttings; cuttings treated with Hormodin 3 gave best rooting.

Larch trees exhibit considerable heterosis as early as their second year and at 10 years of age from seed there is conspicuous segregation into height classes. For example, the height of *Larix decidua* seedlings ranges from 13 to 23 feet; *L. leptolepis* 23 to 38 feet; *L. eurolepis* 23 to 38 feet; and *L. laricina* 6 to 21 feet. Thus, by the time the cuttings become more difficult to root, due to age of the trees, individuals are already well segregated so that superior trees can be selected with a considerable degree of accuracy.

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# BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF TISSUE CULTURES AND THE PLANT PARTS FROM WHICH THEY ARE DERIVED. I. AGAVE TOUMEYANA TREL.<sup>1</sup>

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AND WALTER TULECKE<sup>3</sup>

## SUMMARY

The composition of leaves of *Agave toumeyana* and a tissue culture derived from the leaves were compared for their relative content of free and combined amino acids, nonvolatile organic acids, sugars and nucleic acids.

Outstanding differences were found in the content of free and combined hydroxyproline, the ratios of malate to citrate, of ribonucleic to deoxyribonucleic acid and of free glucose, fructose and sucrose.

## INTRODUCTION

These investigations compare the biochemistry of plant tissue cultures with the biochemistry of the plant parts from which they were derived. Such a comparison affords an opportunity to study gross differences in cellular physiology in an intact, slow-growing, nonsterile, relatively mature organ and analogous rapidly proliferating tissue grown aseptically. Emphasis is placed on analyses of free amino acids, protein amino acids, nonvolatile organic acids, sugars and nucleic acids. Growth data as they correlate with analytical results are included.

Since a tissue culture represents a segment of the whole plant, diverted in its normal function and removed from some of the controls of the parent plant, both similarities and differences are to be anticipated. An understanding of important differences between the tissue culture and the plant is the object of this series of papers. The present paper is concerned with a comparison of a tissue culture from the leaves of *Agave toumeyana* Trel. and the leaves themselves.

*Agave toumeyana* Trel. is a small plant (Fig. 1 A) which occurs only in central Arizona (1, p. 86). It is easily recognized by its small leaves with white zigzag markings along the inner side and white threads along the margins.

The original interest in this plant was due to its content of the steroidal sapogenin, hecogenin (6). In the early days of the study of cortisone precursors from plant materials, this sapogenin was considered one of the more promising sources of the steroid nucleus (20).

Seeds from one of these plants were collected in Arizona and used for

<sup>1</sup> Presented at the Annual Conference of Plant Chemists, Bethesda, Maryland, October 17, 1958.

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<sup>3</sup> Biochemical Research Laboratories, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

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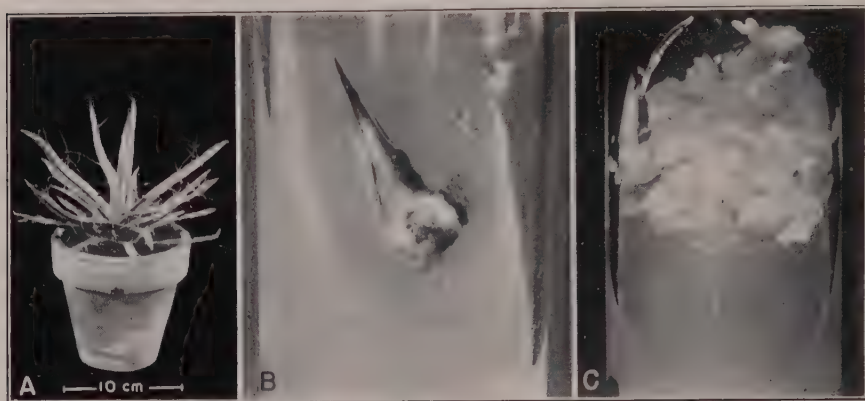


FIGURE 1. *Agave toumeyana*. (A) Seven-year-old greenhouse-grown plant. (B) Callus formation at basal end of excised leaf tip. (C) Leaf tissue culture on White's basal medium supplemented with coconut milk and 2,4-D.

various physiological studies. Seeds from this same batch were germinated in the greenhouse for further use in the study of hecogenin synthesis. Some were germinated aseptically for the isolation of tissue cultures of the leaves for the purpose of studying the *in vitro* synthesis of hecogenin.

Other than the studies concerned with its steroidal content, little is known of the other components of this plant. Srinivasan and co-workers (13, 14, 15) have made an extensive study of the carbohydrates of *Agave vera cruz* Mill. They found sucrose, glucose, fructose, 4 unidentified carbohydrates and polyfructosan. Upon hydrolysis, the juice carbohydrates yielded only glucose and fructose.

#### MATERIALS AND METHODS

*Establishment of tissue culture.* The original isolations from the plant, grown under aseptic conditions were made in 1951. Sections of the aseptic leaves were placed on nutrient medium containing yeast extract and *p*-chlorophenoxyacetic acid. After considerable callus had formed (Fig. 1 B) subcultures were made on this medium. Later, the culture (Fig. 1 C) was transferred to White's basal medium containing coconut milk and 2,4-D. All tissues used for analysis in the present work were grown on the latter medium (7), unless otherwise stated.

The leaves used for comparison with the tissue culture were from mature, greenhouse-grown plants about seven years old. Plants raised aseptically from the same batch of seed were those from which the tissue cultures were derived.

*General characteristics of the tissue culture.* Since the original reason for isolating this culture was to determine whether hecogenin was produced *in vitro*, analyses for this sapogenin have been made many times. At no

time since the isolation of the tissue has hecogenin been demonstrated in the culture.

This tissue culture represents a true tissue, since it is composed only of parenchyma cells (7). The tissue characteristically grows as a plate several cells thick, with a full meristem on each side. When suspended in liquid, these meristems cut off cells on both sides of the plate, contributing to the rapid formation of a culture consisting of lamina of tissue and derived cells.

*Experimental culture techniques and media.* Standard plant tissue culture techniques were employed for all manipulations (23). The general experimental procedures and growth conditions are the same as used previously (8). Comparisons of growth are expressed by the term "growth value" which represents the final wet weight divided by the inoculum weight.

The culture material used for analysis was grown on White's medium (23, p. 103) supplemented by 2,4-D (6 p.p.m.) and coconut milk (18 per cent by volume). This medium is termed W-2,4-D-CM. The coconut milk added to culture media is prepared by collecting the liquid from 100 mature coconuts. This pooled coconut milk is filtered, dispensed into flasks, and autoclaved. Before use, the coconut milk is again filtered to remove precipitated protein. In this processed form it is then added to media at 18 ml. / 100 ml. of medium. The rate of growth of this culture is shown in the upper growth curve in Figure 2. By comparison the lower curve represents the growth of this tissue on a medium in which the coconut milk has been replaced by 0.2 per cent yeast extract, showing a partial substitution for the coconut milk.

In determining the components of a synthetic medium which will support satisfactory growth of the *Agave* culture, many different media were used. One of these was a synthetic medium used to study the response to various sugars. The outstanding differences of this medium from the W-2,4-D-CM were a high level of phosphate (165 p.p.m. of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) and the addition of the amino acid arginine (100 p.p.m. of L-arginine HCl).

*Analytical techniques.* Fresh tissues for analysis (about 20 grams) were weighed and ground in 80 per cent ethanol in a VirTis "45" homogenizer. The tissues were extracted four additional times with 80 per cent ethanol at 50° C. and the supernatants were combined. An aliquot of tissue was dried at 110° C. for two hours for dry weight determination.

The ethanolic extract was passed through a 1 × 5-cm. bed of hydrogen-form Dowex 50-X8, 200 to 400 mesh, to take up free amino acids and amides. The filtrate was then passed through a 1 × 10-cm. bed of acetate-form Dowex 1-X10, 200 to 400 mesh, to take up nonvolatile organic acids. The fraction containing sugars passed through Dowex 1 and was taken to dryness. Pigments and lipoidal substances were removed from the latter fraction by emulsifying in water-chloroform, followed by centrifugation, and removal of the aqueous layer.



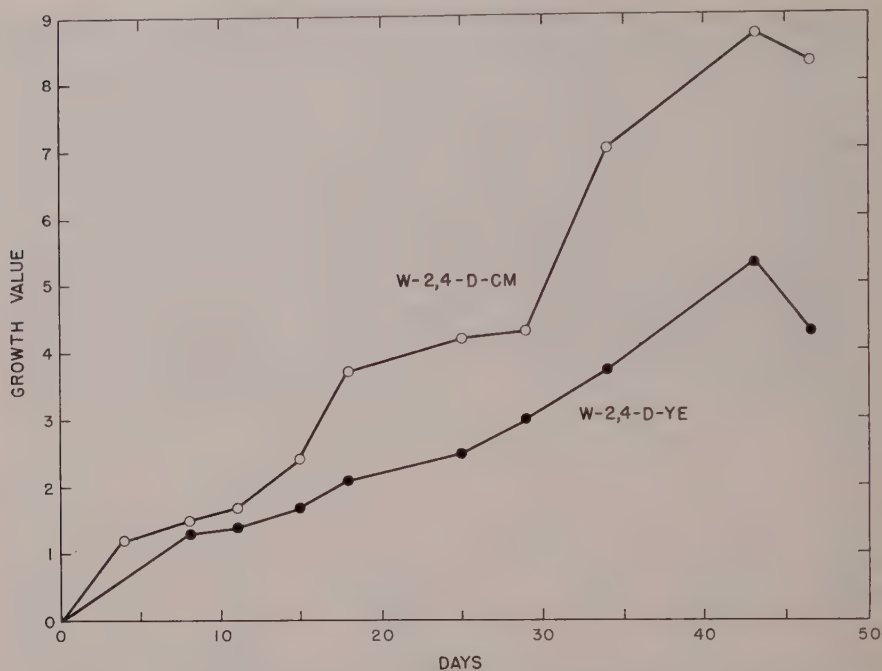


FIGURE 2. Growth curves of the *Agave toumeyana* leaf tissue culture. Growth value, final fresh weight/initial fresh weight; CM, 18 per cent coconut milk; YE, 0.2 per cent yeast extract (Mead-Johnson); 2,4-D, 6 p.p.m. 2,4-dichlorophenoxyacetic acid; W, White's basal medium (23); temperature 25° C.

The alcohol-insoluble residues were lyophilized.

Free amino acids were eluted from Dowex 50 by the method of Plaisted (10), and were analyzed quantitatively by the two-dimensional paper chromatographic procedure of Porter, Margolis and Sharp (12). Organic acids were separated on Dowex 1 by gradient elution with 2.5*N* acetic acid followed by 6*N* formic acid (9, 22). Individual acids were identified by one-dimensional paper chromatography and by infrared absorption spectra. The acids were isolated as described previously (21, 22).

Sugars were analyzed by one-dimensional ascending paper chromatography on Schleicher and Shull No. 598 paper employing triple solvent development in *n*-butanol—acetic acid—water (4:1:5 v/v/v). Guide strips of known sugars and of the tissue extracts were developed with 0.2 per cent naphthoresorcinol in ethanol and phosphoric acid (9:1 v/v; 90° C. for 5 minutes) (4). Corresponding areas were then cut from undeveloped portions of the chromatograms. The spots were eluted with hot water and filtered through glass wool. The eluate was dried under a stream of warm air. Two milliliters of water were added, followed by 4 ml. of 2 per cent

anthrone in concentrated  $\text{H}_2\text{SO}_4$ . The tubes were allowed to stand for ten minutes, and the optical density was read in a Model DU Beckman spectrophotometer at  $640 \text{ m}\mu$  (16).

For protein amino acids, aliquots of the alcohol-insoluble residue were hydrolyzed in  $6N$   $\text{HCl}$  for 15 hours. The hydrolyzate was taken to dryness several times on a steam bath, was taken up on hydrogen-form Dowex 50-X8, and eluted with  $4N$  aqueous  $\text{NH}_4\text{OH}$ . The amino acids were then determined by two-dimensional paper chromatography. Total nitrogen of the hydrolyzate was determined by the semi-micro Kjeldahl procedure.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined by differential perchloric acid extraction (5) followed by phosphorus determination (11).

### RESULTS

The data have been calculated on the conventional bases of fresh weight and dry weight, and also on the basis of DNA-phosphorus. The latter mode of expression is included to attempt a more accurate interpretation at the cellular level, dependent as it is on the percentage of nucleate cells and nuclear size.

Results of RNA-phosphorus and DNA-phosphorus analyses are shown in Table I. It is clear that the tissue culture was very high in DNA as compared with leaves. This is borne out by RNA-phosphorus/DNA-phosphorus ratio of 4.1 in leaves, and the extremely low ratio of 2.0 in tissue cultures. The ratio for *Agave* leaves is somewhat low when compared with other plant materials (e.g., Paul's Scarlet rose leaves, 27.0; Paul's Scarlet rose stem tissue culture, 13.6). The high DNA in tissue cultures is indicative of the large nuclear volume of proliferating cells with large nuclei.

Of the twelve nonvolatile organic acids separated by ion-exchange and paper chromatography, four acids were positively identified: succinate, malate, citrate and fumarate (Fig. 3). The latter three were the major acids of both leaves and leaf tissue cultures (Table II). In leaf tissues, the dominant acid was found to be malate, the malate/citrate ratio being 4.6. In tissue cultures, however, citrate was the dominant acid, the ratio of malate to citrate being 0.67. The total organic acidity of leaves expressed on a fresh weight basis was about twice that of the tissue culture. On a dry weight basis, the tissues each contained about the same amount of total acidity. On a DNA-phosphorus basis, leaves contained about 6-fold more total acidity than did the tissue cultures. This is not surprising since leaf tissues are more vacuolated and would be expected to contain more water-soluble constituents than the more juvenile tissue culture cells.

*Agave* leaves and leaf tissue cultures were found to contain the usual spectrum of free amino acids and amides with the exception of the presence

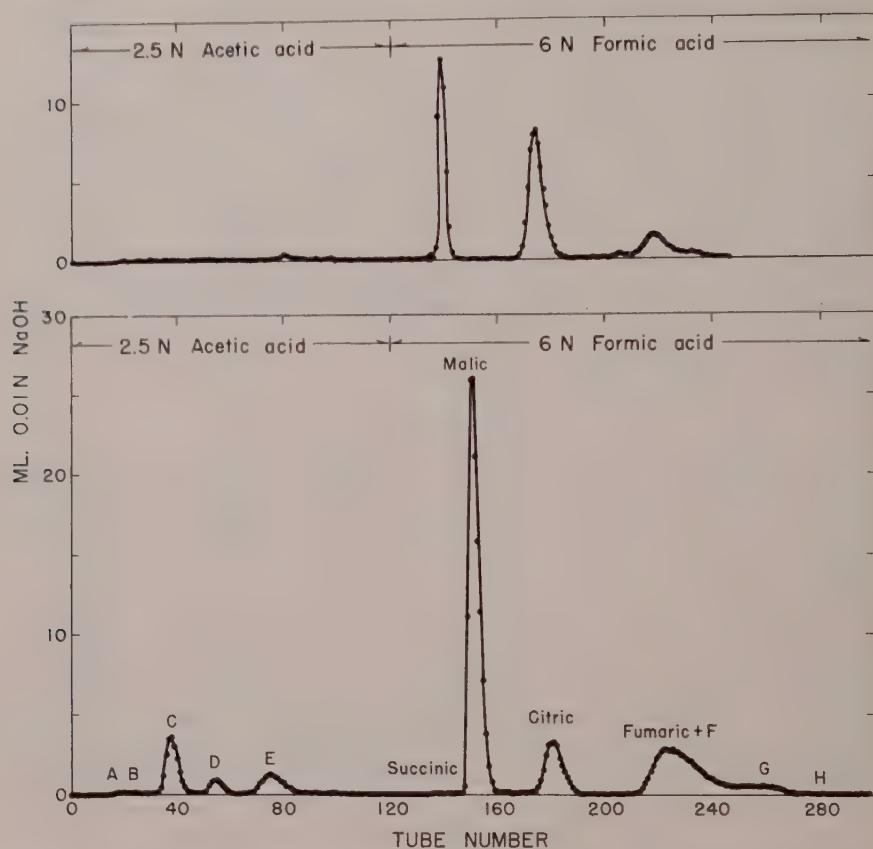


FIGURE 3. Titration curves of organic acids, eluted from Dowex 1-X, 10 resin, of 20 g. of *Agave toumeyana* leaves (lower curve) and leaf tissue culture (upper curve).

TABLE I  
RNA-PHOSPHORUS AND DNA-PHOSPHORUS OF *AGAVE TOUMEYANA*  
LEAVES AND LEAF TISSUE CULTURES

|                | $\mu\text{G. P/g.}$ |                |         |                | Ratio<br>RNA-P/DNA-P |                |
|----------------|---------------------|----------------|---------|----------------|----------------------|----------------|
|                | Fresh wt.           |                | Dry wt. |                |                      |                |
|                | Leaves              | Tissue culture | Leaves  | Tissue culture | Leaves               | Tissue culture |
| RNA-P          | 34.0                | 49.8           | 170.9   | 532.6          | 4.1                  | 2.0            |
| DNA-P          | 8.2                 | 24.5           | 41.2    | 262.0          |                      |                |
| Nucleic acid-P | 42.2                | 74.3           | 212.1   | 794.6          |                      |                |

TABLE II  
NONVOLATILE ORGANIC ACIDS OF AGAVE TOUMEYANA LEAVES  
AND LEAF TISSUE CULTURES

| Organic acid         | $\mu$ Eq./g. |                |         |                | $\mu$ Eq./mg<br>DNA-phosphorus |                |
|----------------------|--------------|----------------|---------|----------------|--------------------------------|----------------|
|                      | Fresh wt.    |                | Dry wt. |                |                                |                |
|                      | Leaves       | Tissue culture | Leaves  | Tissue culture | Leaves                         | Tissue culture |
| A                    | 0.13         | 0.38           | 0.65    | 4.06           | 15.9                           | 15.5           |
| B                    | 0.09         | 0.56           | 0.43    | 5.99           | 1.1                            | 22.8           |
| C                    | 9.95         | 0.37           | 50.00   | 3.96           | 1217.0                         | 15.1           |
| D                    | 3.04         | 0.88           | 15.27   | 9.41           | 372.0                          | 35.9           |
| E                    | 3.74         | 1.65           | 18.78   | 17.65          | 457.0                          | 67.3           |
| Succinic             | 0.36         | —              | 1.78    | —              | 44.0                           | —              |
| Malic                | 63.54        | 19.08          | 319.00  | 204.06         | 7780.0                         | 778.0          |
| Citric               | 13.72        | 28.34          | 68.90   | 393.10         | 1680.0                         | 1155.0         |
| Fumaric +<br>unknown | 26.59        | 10.14          | 133.50  | 108.45         | 3250.0                         | 414.0          |
| F                    | 5.82         | —              | 29.20   | —              | 712.0                          | —              |
| G                    | 0.43         | —              | 2.14    | —              | 52.6                           | —              |
| Total                | 127.41       | 61.40          | 639.65  | 656.68         | 15,581.6                       | 2503.6         |

of hydroxyproline in the tissue culture and of a number of unidentified amino compounds in both the leaf and the tissue (Table III). On a fresh weight basis, both contained approximately equivalent amounts of amino compounds. On a dry weight basis, the tissue culture contained about twice as much as leaves, and on a DNA-phosphorus basis, leaves were about 3-fold higher than the tissue culture. The dominant amino compounds in leaves were glutamate and glutamine, while in the tissue culture they were glutamate, serine, alanine and arginine.

Of the free sugars present in the tissues, sucrose, glucose and fructose were analyzed quantitatively. Various concentrations of six unidentified sugars or sugar polymers were also present, all of which reacted as ketoses with the naphthoresorcinol-phosphoric acid reagent. One of these has been identified as raffinose while the others are presumably fructosans as reported by Bhatia *et al.* (2). On a fresh weight basis, both leaves and tissue cultures contained approximately equal amounts of the sugars determined (Table IV). On a dry weight basis, the tissue culture had about twice as much total sugar. In leaves, sucrose and glucose were present in approximately equal amounts, with fructose at a lower concentration. In the tissue culture, however, the dominant sugar was fructose, followed by glucose, and finally, by sucrose.

The growth response of the tissue culture to these sugars shows a direct correlation between their utilization as carbohydrate sources and their presence in the tissue (Fig. 4). The growth response of *Agave* tissue culture on synthetic medium, consisting of W-2,4-D (minus the coconut milk), increased phosphate, and arginine, was compared to growth on this same



TABLE III  
FREE AMINO ACIDS OF AGAVE TOUMEYANA LEAVES  
AND LEAF TISSUE CULTURES

| Amino acids*           | $\mu\text{G./g.}$ |                |         |                | Mg./mg.<br>DNA-phosphorus |                |
|------------------------|-------------------|----------------|---------|----------------|---------------------------|----------------|
|                        | Fresh wt.         |                | Dry wt. |                | Leaves                    | Tissue culture |
|                        | Leaves            | Tissue culture | Leaves  | Tissue culture |                           |                |
| Aspartic               | 33.4              | 9.4            | 167.8   | 100.5          | 4.1                       | 0.4            |
| Glutamic               | 39.6              | 31.9           | 199.0   | 341.2          | 4.8                       | 1.3            |
| Serine                 | 26.7              | 36.2           | 134.2   | 387.2          | 3.3                       | 1.5            |
| Glycine                | 9.0               | 3.8            | 45.2    | 40.6           | 1.1                       | 0.2            |
| Threonine              | 10.9              | 6.5            | 54.8    | 69.5           | 1.3                       | 0.3            |
| Alanine                | 15.7              | 22.9           | 78.9    | 244.9          | 1.9                       | 0.9            |
| Lysine                 | 4.4               | 4.5            | 22.1    | 48.1           | 0.5                       | 0.2            |
| Arginine               | 9.3               | 9.6            | 46.7    | 102.7          | 1.1                       | 0.4            |
| Proline                | Trace             | Trace          | Trace   | Trace          | Trace                     | Trace          |
| Valine                 | 11.8              | 9.8            | 59.3    | 104.8          | 1.4                       | 0.4            |
| Leucines               | 15.1              | 18.3           | 75.9    | 195.7          | 1.8                       | 0.7            |
| Phenylalanine          | 11.6              | 13.0           | 58.3    | 139.0          | 1.4                       | 0.5            |
| Tyrosine               | 6.3               | 3.5            | 31.7    | 37.4           | 0.8                       | 0.1            |
| $\gamma$ -Aminobutyric | 4.0               | 5.6            | 20.1    | 59.9           | 0.5                       | 0.2            |
| Hydroxyproline         | —                 | 5.6            | —       | 59.9           | —                         | 0.2            |
| Methionine             | 6.5               | 8.0            | 32.7    | 85.6           | 0.8                       | 0.3            |
| Asparagine             | Trace             | 5.2            | Trace   | 55.6           | Trace                     | 0.2            |
| Glutamine              | 55.8              | 10.2           | 280.4   | 109.8          | 6.8                       | 0.4            |

\* Eleven unidentified amino compounds also present.

medium with various sugars substituted for the sucrose. All the sugars were run at 2 per cent concentration. Fructose, glucose, raffinose and sucrose, in that order, served as good carbohydrate sources. Xylose, galactose and lactose were utilized to a slight extent, but the tissue died on arabinose. No growth took place on sorbitol, although the tissue was not killed. Inositol allowed some growth, but it was less than that of any sugar,

TABLE IV  
SUGARS OF AGAVE TOUMEYANA LEAVES AND LEAF  
TISSUE CULTURE

|          | Mg./g.    |                |         |                | Mg./mg.<br>DNA-phosphorus |                |
|----------|-----------|----------------|---------|----------------|---------------------------|----------------|
|          | Fresh wt. |                | Dry wt. |                | Leaves                    | Tissue culture |
|          | Leaves    | Tissue culture | Leaves  | Tissue culture |                           |                |
| Sucrose  | 3.23      | 1.25           | 16.21   | 13.37          | 395.0                     | 51.0           |
| Glucose  | 3.11      | 2.48           | 15.63   | 26.52          | 380.0                     | 101.2          |
| Fructose | 1.77      | 3.59           | 8.88    | 38.40          | 216.5                     | 146.5          |
| Total    | 8.11      | 7.32           | 40.72   | 78.29          | 991.5                     | 298.7          |

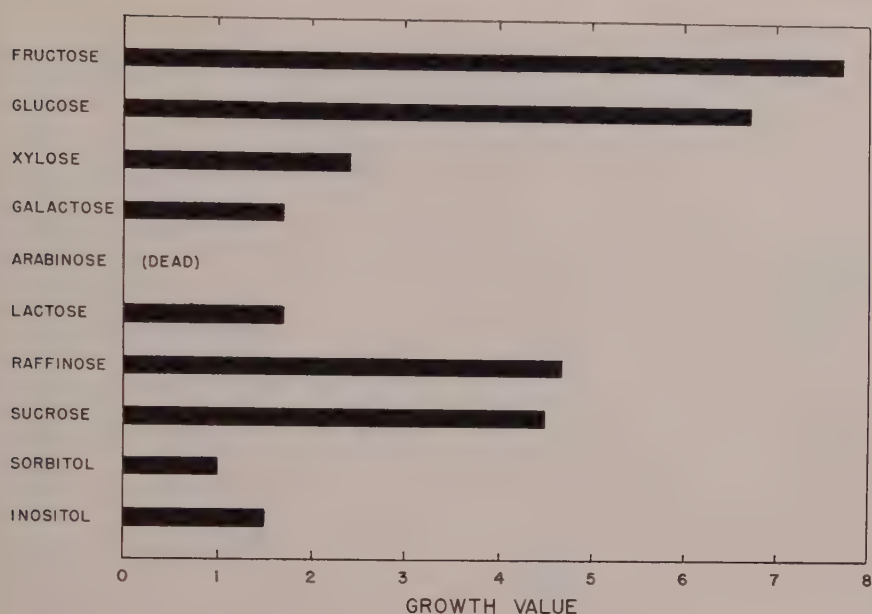


FIGURE 4. The utilization of various sugars and sugar alcohols by *Agave toumeyana* leaf tissue culture. The synthetic medium contained essentially White's basal medium (23); 2,4-D, 6 p.p.m. 2,4-dichlorophenoxyacetic acid; L-arginine HCl, 100 p.p.m.; and increased phosphate,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 165 p.p.m. The sucrose of White's medium was replaced by the other sugars at 2 per cent concentration. The growth value equals the final fresh weight/initial fresh weight. The tissue dies in the absence of carbohydrate.

with the exception of arabinose. It should be kept in mind that the level of inositol used was probably abnormally high since it is usually considered as a growth factor.

Data for bulk protein amino acids are shown in Table V. Cysteic acid and histidine were not detected in leaf tissues. Although certain losses are inherent in acid hydrolysis of proteins, especially in the presence of large amounts of carbohydrate materials (3, pp. 22-27), obvious differences in protein composition were evident. The tissue culture protein was found to be richer than leaf protein in lysine, phenylalanine, tyrosine and hydroxyproline. The leaf protein was richer in alanine, valine, methionine and proline.

#### DISCUSSION

The transition from relatively slow-growing *Agave* leaf tissues to rapidly-growing tissue cultures exerts a profound influence on the chemical composition, physiology and cytology of the tissues. This is borne out by a number of facts. The high DNA content of the tissue cultures confirms the

TABLE V  
PROTEIN AMINO ACIDS OF AGAVE TOUMEYANA LEAVES AND LEAF  
TISSUE CULTURE

| Amino acids          | Amino acid N as<br>% of protein N |                   | Molar ratio |                   |
|----------------------|-----------------------------------|-------------------|-------------|-------------------|
|                      | Leaves                            | Tissue<br>culture | Leaves      | Tissue<br>culture |
| Cysteic              | —                                 | 1.65              | —           | 0.30              |
| Aspartic             | 3.80                              | 3.85              | 0.78        | 0.88              |
| Glutamic             | 6.98                              | 6.40              | 1.28        | 1.32              |
| Serine               | 7.00                              | 6.67              | 1.79        | 1.93              |
| Glycine              | 6.99                              | 6.19              | 2.51        | 2.51              |
| Threonine            | 4.44                              | 3.92              | 1.00        | 1.00              |
| Alanine              | 10.18                             | 7.55              | 3.08        | 2.57              |
| Histidine            | —                                 | 5.90              | —           | 0.38              |
| Lysine               | 10.57                             | 14.31             | 0.98        | 1.48              |
| Arginine             | 13.88                             | 11.88             | 0.53        | 0.52              |
| Proline              | 4.06                              | 2.30              | 0.95        | 0.60              |
| Valine               | 6.77                              | 4.99              | 1.57        | 1.29              |
| Leucines             | 14.65                             | 11.73             | 3.00        | 2.71              |
| Phenylalanine        | 2.60                              | 3.29              | 0.42        | 0.61              |
| Tyrosine             | 2.49                              | 2.94              | 0.37        | 0.49              |
| Hydroxyproline       | 3.37                              | 5.06              | 0.69        | 1.18              |
| Methionine sulfoxide | 2.20                              | 1.32              | 0.40        | 0.27              |

cytological observations of large numbers of proliferating cells with large nuclei. Analyses of nonvolatile organic acids show a striking quantitative change in the proportions of the acids present, especially of malate and citrate. Conversion of normal *Agave* leaf tissue to a heterotrophic tissue culture induces a change from a "malate" tissue to a "citrate" tissue. Although the cause of this phenomenon is not known, it has often been observed that marked changes in the ratios of the dominant organic acids occur. An outstanding example of a citrate to malate shift has been observed in the case of Red Pontiac potato tubers and their tissue cultures. In some cases, the shift is less prominent (*Dioscorea composita* Hemsl. tuber and its tissue culture) but nevertheless exists.

In order to determine if the malate-citrate content of the added coconut milk is directly responsible for the levels of these acids in the tissue culture, preliminary analyses were made for the nonvolatile organic acids in the processed coconut milk. Based on the levels found, calculations showed that the amount present in each tube of medium was about 4 mg. of malic acid and about 0.7 mg. of citric acid. Thus, at the initiation of growth, the tissues were exposed to a medium which had a ratio of malic/citric of about 6, a ratio similar to that found in normal *Agave* leaves. Upon harvest of the tissue cultures, however, the ratio was found to be about

0.7 (Table II). It is clear, then, that the organic acids in the medium are not responsible for the final malic/citric ratio of the tissue cultures. The final ratio is probably characteristic of the tissue and not of the medium.

Based on biochemical analyses it can be concluded that the tissue culture is cytologically, physiologically and biochemically distinct from the parent plant part.

Steward *et al.* (19) have reported that rapidly proliferating excised potato tuber tissue has a much lower content of free amino acids than the resting, nongrowing excised tuber tissue when expressed on a fresh weight basis. In the case of *Agave*, this is true only when the data are expressed on a DNA-phosphorus basis. When expressed by other means there is either little change in the free amino acid content (fresh weight basis) or the tissue culture is markedly higher (dry weight basis). In addition, free hydroxyproline was present in the tissue culture but not in the leaves, even though its occurrence in the free state is considered to be rare (18). The data for protein amino acids are partially in agreement with those reported by Steward *et al.* (19) for proteins of potato tuber, carrot root, and their derived tissue cultures, for leaves and crown gall tumors of *Kalanchoe daigremontiana* Hamet & Terrier, and for tobacco leaves and genetic leaf tumors. The points of general agreement include the data for hydroxyproline and phenylalanine, which, in most instances, were found by them to increase in rapidly-proliferating tissues. Not in agreement, however, are the results for lysine, proline and tyrosine. In the case of proline and hydroxyproline, results shown in Table V indicate that in the transformation of leaf to the leaf tissue culture, the bulk protein becomes richer in hydroxyproline, and that the proline content decreases an equivalent amount. Thus, the results suggest that the transformation in *Agave* from slow-growing to rapidly-proliferating tissues involves a conversion of proline to hydroxyproline in protein synthesis. The conversion of proline to hydroxyproline in carrot tissue explants has been demonstrated by Steward and Pollard (17).

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# CHARACTERIZATION OF VANILLA AND OTHER PLANT EXTRACTS BY PAPER CHROMATOGRAPHY<sup>1</sup>

H. P. BURCHFIELD AND EDWARD A. PRILL

## SUMMARY

Vanilla and other plant extracts were applied to Whatman No. 1 filter paper and chromatographed in one dimension with a solvent containing potassium hydroxide (20 g.), sodium bromide (100 g.), and ethanol (200 ml.) made up to 1 liter with water, a solvent containing isopropanol (240 ml.), concentrated ammonium hydroxide (150 ml.), sodium bromide (84 g.), and water (450 ml.), and a solvent containing acetic acid, hydrochloric acid, and water in a 30:3:10 ratio. When viewed under ultraviolet light, fluorescent patterns were seen that were characteristic of the plant extracts. This provided a method for identifying materials of botanical origin that was found to be useful for characterizing vanilla extracts and establishing the presence of many adulterants. In addition, the intensity of fluorescence was proportional to concentration so that dilution of the product could be detected. Four out of seven samples of household vanilla extracts that were examined were obviously diluted, and the patterns of the other three were somewhat weaker than usually obtained on authentic vanilla extract. Seven out of 13 industrial tenfold extracts were adulterated, four were suspected of adulteration, one was grossly diluted, and one gave an authentic but slightly weak fluorescent pattern.

## INTRODUCTION

Authentic singlefold vanilla extract is the extract of 10 grams of the cured unripe fruit of *Vanilla planifolia* (Andrews) or *V. tahitensis* Moore in 100 ml. of solution (4, p. 1266). The solvent is usually 35 per cent alcohol, but it may also contain added sucrose, glycerine, or propylene glycol. The tenfold vanilla extracts used in the baking and dairy industries are supposed to be singlefold extracts concentrated to one-tenth the original volume. However, because of the high and fluctuating price of vanilla beans, some manufacturers market products which are adulterated with foreign botanicals to increase color, lead number, and resin content. Many of these malpractices can be detected by quantitative methods of analysis (5), but the need still exists for a simple screening procedure by which large numbers of samples can be sorted according to quality in advance of more detailed evaluation. This can be accomplished by paper chromatography.

## MATERIALS AND METHODS

*Extracts and reagents.* Commercial tenfold and household vanilla extracts and fluid extracts of hickory bark, angostura, St. John's-wort,

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cherry bark, black walnut, cascara, etc. containing the soluble components of 100 g. of the products in 100 ml. of solution were supplied by the Scientific Research Committee of the Flavoring Extract Manufacturers' Association. Plant extracts other than vanilla were tested at a concentration of 10 per cent of the fluid extract in aqueous alcohol. The preparation of authentic vanilla extracts and manufacturing-type vanilla extracts has been described elsewhere (1).

The principal solvent used for chromatography contained 20 g. of KOH, 100 g. of NaBr and 200 ml. of absolute ethanol diluted to 1 liter with water. With the exception of the NaBr, which was added to decrease the rate of travel of the solvent front, this combination was identical to a mixture suggested by Mitchell.<sup>2</sup> The isopropanol-NaBr-ammonia solvent contained 240 ml. of isopropanol, 150 ml. of concentrated  $\text{NH}_4\text{OH}$ , 84 g. of NaBr, and 450 ml. of water. The third solvent contained acetic acid, hydrochloric acid (37 per cent), and water in a 30:3:10 ratio (2).

The reagent used for extracting the fluorescent pigments from vanilla was a 2 per cent solution of *N,N*-dimethyldodecylamine (Eastman P5829) in chloroform.

Chromatograms were viewed in a dark room using long wavelength ultraviolet Model XX15 Black ray lamps. These are obtainable from Ultra-Violet Products Inc., San Gabriel, Calif.

*Procedure.* Samples of vanilla extract at fivefold strength were applied to  $18\frac{1}{8} \times 22\frac{1}{4}$  inch Whatman No. 1 paper as described previously (2). To examine single-strength extracts containing sucrose and other additives such as glycerine and propylene glycol, the extracts were concentrated on the paper by applying five aliquots of 10 $\mu$  each to the same location, the paper being dried carefully between applications with a hair drier to prevent spreading of the spots. The samples were spaced 2.5 inches apart in a row parallel to the long edge ( $22\frac{1}{4}$  inches) of the paper, and development with solvent was carried out perpendicular to the machine direction of the paper.

The same equipment and techniques of chromatography described earlier were employed (2) except that the ascending method was used when the solvent was KOH-NaBr-ethanol-water. To accomplish this the troughs containing the solvent were placed on blocks  $2\frac{3}{4}$  inches high located at each end of the bottom of the cabinet. The papers were attached to glass rods with small wooden clothes pins, and the ends of the rods were supported on the metal strips attached to the two ends of the cabinet in such a way that the bottom edges of the papers were immersed in solvent. One paper was used per trough, and six troughs per box. Six open Petri dishes containing solvent were placed on the bottom of the chromatograph-

<sup>2</sup> Mitchell, Lloyd C. Personal communication.

ic cabinet to maintain a semi-saturated atmosphere, and the lid of the cabinet was sealed with masking tape. The cabinets were opened after 22 hours. Occasionally it was necessary to examine the papers under fluorescent light before they were dried to avoid missing some details which faded on longer standing. In cases where the papers were rechromatographed in the same solvent, 16 hours were sufficient for the second development.

Samples which contained added sucrose were sometimes extracted with chloroform-amine reagent to avoid the retarding effects of this material on  $R_f$  values. A 10-ml. sample of vanilla at singlefold strength was extracted with 2 ml. of the reagent, and 10 $\lambda$  of the lower (chloroform) layer spotted on paper. For more detailed examinations three extractions were made and the combined lower layers evaporated and redissolved in 2 ml. of chloroform. The supernatant alcohol-water mixture was next evaporated and redissolved in 2 ml. of 50 per cent ethanol. A 10 $\lambda$  aliquot of each fraction was then spotted on paper and chromatographed.

#### RESULTS ON VANILLA AND OTHER PLANT EXTRACTS

When vanilla and other plant extracts are chromatographed on paper and examined under ultraviolet light, patterns are obtained that can be used as criteria of identity. Whatman No. 1 filter paper is used, while the most useful solvents so far discovered are KOH-NaBr-ethanol, isopropanol-NaBr-ammonia, and acetic acid-hydrochloric acid-water, in that order. The usual procedure is to spot 10 $\lambda$  of a fivefold concentrate of vanilla extract on paper and chromatograph with one or the other of these solvents by the ascending or descending method.

When viewed under ultraviolet radiation, authentic vanilla extracts prepared from Mexican and Bourbon beans give chromatographic patterns that differ only in minor detail (Fig. 1). Tahiti extract contains a material which gives rise to a bright blue fluorescent spot at  $R_f$  0.7 when the paper is developed with KOH-NaBr-ethanol. The color of this spot is not so intense in other extracts. Vanilla prepared from Tahiti beans can also be distinguished from other extracts by chromatographing in isobutanol-acetic acid (2) or by gas chromatography (1).

A schematic diagram of the fluorescent pattern obtained on chromatographing vanilla extract with KOH-NaBr-ethanol is shown in Figure 2. This pattern with minor variations was produced by 20 extracts prepared from vanilla beans representing the principal growing areas of the world, as well as by a large number of extracts believed to be authentic but which were prepared in other laboratories (5).

Region *I* is an oblong spot with an  $R_f$  value of 0.02. It is white in color, fluoresces with moderate intensity and is present in all authentic vanilla extracts that were tested. Region *II* is a diffuse white streak that fluoresces



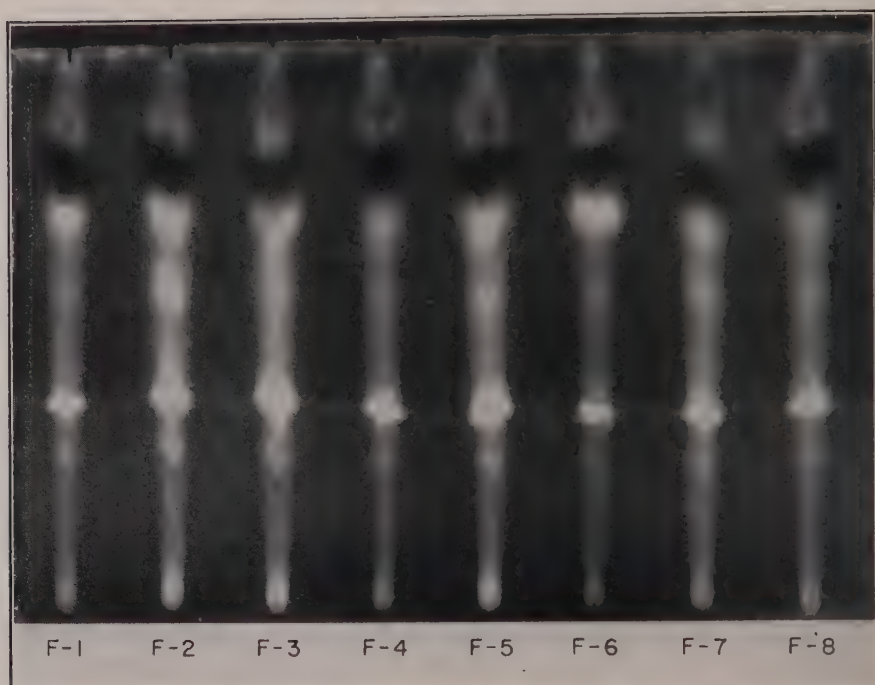


FIGURE 1. Chromatographic patterns developed with KOH-NaBr-ethanol obtained on eight authentic vanilla extracts prepared from (F-1) Madagascar thirds, (F-2) Guadeloupe beans, one-half whole and one-half splits, (F-3) Mexican medianas, (F-4) Reunion seconds, (F-5) Comores thirds, (F-6) Tahiti, one-half white label and one-half yellow label, (F-7) split Bourbon fourths, new crop, and (F-8) whole Bourbon thirds, new crop.

with low intensity. The spot *III* at  $R_f$  0.27 is white. Although weak, it is well defined and is present in all authentic Mexican and Bourbon extracts. However, it is barely distinguishable from background fluorescence in extracts of Tahiti beans. The zone *IV* is diffuse and exhibits fluorescence similar to *II*. Regions *I'* and *IV'* are brilliant orange to orange yellow bands with  $R_f$  values in the neighborhood of 0.36. Many plants contain components which fluoresce in this region, and in some cases the emitted light is brilliant blue in color. Sometimes these materials are easily seen in the presence of vanilla, but more often it is necessary to develop the chromatogram a second time in the same solvent to obtain satisfactory resolution in this region. These components are always present in authentic vanilla extracts, and they fluoresce with high intensity. When they are absent or fluoresce weakly, it is almost certain that a full weight of vanilla beans has not been used. Region *VII* is a zone of diffuse fluorescence which is more intense than *II* and *IV*. Region *VIII* appears as a moderately intense white spot with an  $R_f$  of 0.57. It is present in most authentic extracts,

but in some cases it is barely distinguishable from the diffuse fluorescent zones above and below it. The resolution of *VIII* can be improved by extracting the fluorescent pigments from the vanilla with chloroform-amine reagent before spotting them on paper. Apparently this solvent removes the compound responsible for the fluorescence at *VIII* more readily than it does the materials responsible for the background. This results in better contrast (Fig. 3). *IX* is a region of strong general fluorescence while *X* is a blue spot. Sometimes *X* is not resolved from *XI*, and it is necessary to develop the paper a second time. As mentioned before, *X* is very brilliant on chromatograms of Tahiti extracts, but it is uncertain whether it arises from the same compound that appears at this Rf value with Mexican and Bourbon extracts. Although *X* is not resolved in all authentic extracts, its presence is a good indication that vanilla beans have been used in substantial amounts. *XI* is a blue-black spot which is caused by the absorption of light by vanillin. This has been demonstrated by co-chromatography with a known sample of this chemical. *XI* serves as a useful landmark, but its presence has no special significance in assessing quality since it can be added at will to extracts. Region *XII* is a diffuse spot with very weak white fluorescence, while *XIII* is a compact brown spot visible under ordinary illumination. Sometimes *XIII* is not observed in samples having high sugar content. In such instances it can often be resolved by developing the paper a second time in the same solvent.

| Region Number | Chromatogram | Approximate Rf | Color Under Ultra Violet Radiation | Remarks                                                 |
|---------------|--------------|----------------|------------------------------------|---------------------------------------------------------|
| XIII          |              | 0.98           | Brown (visible)                    | Sometimes unresolved in the presence of sugar           |
| XII           |              | 0.87-0.96      | White                              | Diffuse and very faint                                  |
| XI            |              | 0.78           | Blue-black                         | Absorbent spot arising from vanillin                    |
| X             |              | 0.71           | Blue                               | Sometimes unresolved from XI, intense in Tahiti         |
| IX            |              | 0.61-0.69      | Yellow-white to yellow-green       | Present in all authentic                                |
| VIII          |              | 0.57           | White                              | Sometimes indistinguishable from background             |
| VII           |              | 0.37-0.53      | Yellow-white to yellow-green       | Present in all authentic                                |
| V VI          |              | 0.36           | Yellow/orange                      | V & VI sometimes unresolved, especially on small papers |
| IV            |              | 0.30-0.35      | White                              | Present in all authentic                                |
| III           |              | 0.27           | White                              | Faint but always present except for Tahiti              |
| II            |              | 0.07-0.24      | White                              | Diffuse                                                 |
| I             |              | 0.02           | White                              | More brilliant than II                                  |

FIGURE 2. Schematic diagram of composite chromatogram obtained on authentic vanilla extracts using KOH-NaBr-ethanol for development.

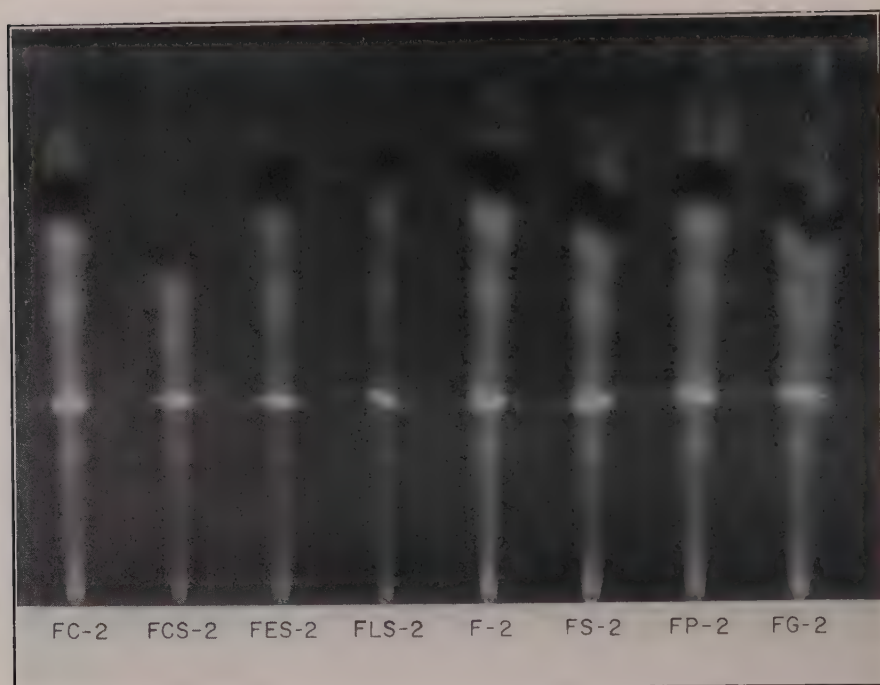


FIGURE 3. Chromatographic patterns obtained on Guadeloupe extract when applied to paper in different ways and developed with KOH-NaBr-ethanol. FC-2 applied as a fivefold concentrate without added sucrose, FCS-2 applied as a fivefold concentrate with added sucrose, FES-2 applied as a chloroform-amine extract, FLS-2 pigments isolated by precipitation with basic lead acetate, F-2 applied as singlefold without sucrose by multiple spotting, FS-2 applied as singlefold with sucrose by multiple spotting, FP-2 contained 20 per cent propylene glycol and was applied by multiple spotting, FG-2 contained 20 per cent glycerol and was applied by multiple spotting.

Good separation of some of the components of vanilla extracts can be obtained in other solvents. Thus isopropanol-NaBr-ammonia gives five distinct fluorescent spots as well as the absorbing spot derived from vanillin. Acetic acid-hydrochloric acid gives two very well resolved spots with low  $R_f$  values and one large streak with a high  $R_f$  value with all authentic vanilla extracts (Fig. 4). The position of the upper streak in this pattern is markedly influenced by the presence of added sugar. In the absence of sugar it is more nearly circular and runs closer to the solvent front.

It should be stressed that these tests provide a means of establishing whether vanilla beans have been used in reasonable amounts in preparing extracts. It has been demonstrated repeatedly on authentic samples that the resolution obtainable with these solvent combinations is sufficient to do this. If a commercial extract is chromatographed in two or three solvents and found to contain most of the components present in authentic

extracts, it is fair to assume the product was prepared from vanilla beans.

Several permissible additives which are frequently used in preparing household vanilla extracts may interfere with the results of the test unless preventive steps are taken. These are propylene glycol, glycerine, and sugar. They may interfere in two ways. Sugar tends to decrease  $R_f$  values. Thus the blue-black vanillin spot in FC-2 (Fig. 3), a concentrate with no added sugar, is moved farther up the paper during chromatography than the vanillin spot in FCS-2, an extract prepared with added sugar. Furthermore, none of these additives are volatile, so it would be impossible to test all commercial samples at equal strength by evaporating the water and alcohol and dissolving the residues in a standard volume of solvent.

This potential concentration error can be eliminated by spotting single-strength vanilla extract on paper in five portions of 10 $\mu$ l each, the paper being dried between applications with a hair drier. In this way it is possible to secure a fivefold concentration of the extract directly on the paper and so avoid misjudging the quality of a product because of the presence of a nonvolatile additive. As can be seen (Fig. 3), propylene glycol (FP-2) and glycerine (FG-2) when used in authentic extracts at concentrations of

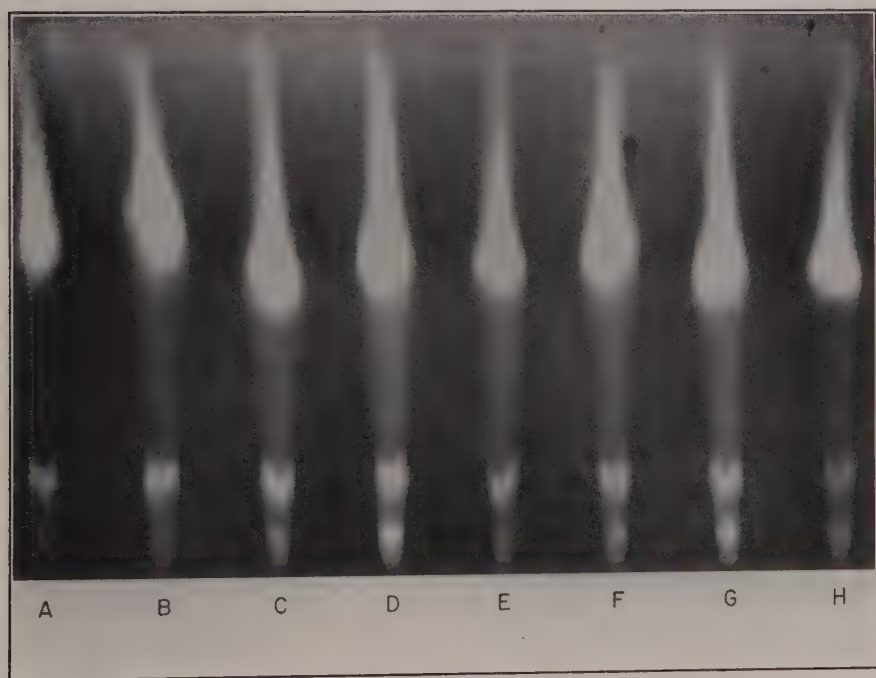


FIGURE 4. Chromatographic patterns obtained on authentic vanilla extracts containing added sugar when developed with acetic acid-hydrochloric acid. A. Mexican buena cuts, B. Guadeloupes (half splits, half cuts), C. Bourbon thirds, D. Reunion, E. Mexican medianas, F. Comores, G. Tahiti, and H. Dominican beans (5).



20 per cent did not cause any reduction in intensity of fluorescence or loss in detail when the extracts containing them were applied to paper in this way and chromatographed. Some broadening of the pattern occurred, but this can be reduced to a minimum if the paper is dried very carefully between applications.

The multiple spotting method also reduces the retarding effect of sucrose on  $R_f$  values (Fig. 3). For example, the vanillin spot obtained on chromatographing a fivefold vanilla concentrate containing added sugar (FCS-2) had a somewhat lower  $R_f$  value than the vanillin spot found on a single-strength vanilla containing sugar which was concentrated on the paper by multiple spotting (FS-2). The retarding effect of sugar can be reduced further by developing the paper a second time in the same solvent. If the  $R_f$  value of vanillin still is not the same as that of the standard sample, the fluorescent pigments can be extracted from the vanilla with chloroform-amine reagent. This process leaves the sugar and other aqueophilic compounds in the water-alcohol phase. The resolution obtained on chromatographing an aliquot of the chloroform layer is excellent (FE-2). However, some pigments which are contained in botanicals used to adulterate vanilla are not readily removed by this reagent. Therefore, it is best to extract three times and chromatograph the combined chloroform layers and the concentrated supernatant to verify the presence of an authentic vanilla pattern and at the same time avoid missing any adulterants that might be present.

In summary, the quality of vanilla extract can be judged with ease and accuracy if the following systematic procedure is used.

1. Dilute tenfold vanilla extracts to fivefold with 50 per cent ethanol and chromatograph on Whatman No. 1 paper with KOH-NaBr-ethanol. Compare with the results obtained on an authentic tenfold.
2. Apply singlefold vanilla extracts to the paper by the multiple spotting method and chromatograph in the same way, using an authentic vanilla extract which does not contain sugar for comparison. If the  $R_f$  of the vanillin spot is lower than the standard, rechromatograph in the same solvent.
3. If the  $R_f$  of the vanillin spot is still too low, extract the vanilla with chloroform-amine reagent and apply both the extract and the supernatant to the paper.
4. If suspicion is aroused concerning the strength of the vanilla or the presence of foreign botanicals, rechromatograph the suspected materials in isopropanol-NaBr-ammonia and/or acetic acid-hydrochloric acid-water.

Originally it was believed possible that this test might be so sensitive to differences in processing methods that reputable manufacturers might not have reasonable leeway in selecting industrial extraction procedures. To determine whether this was true, vanilla beans were extracted under

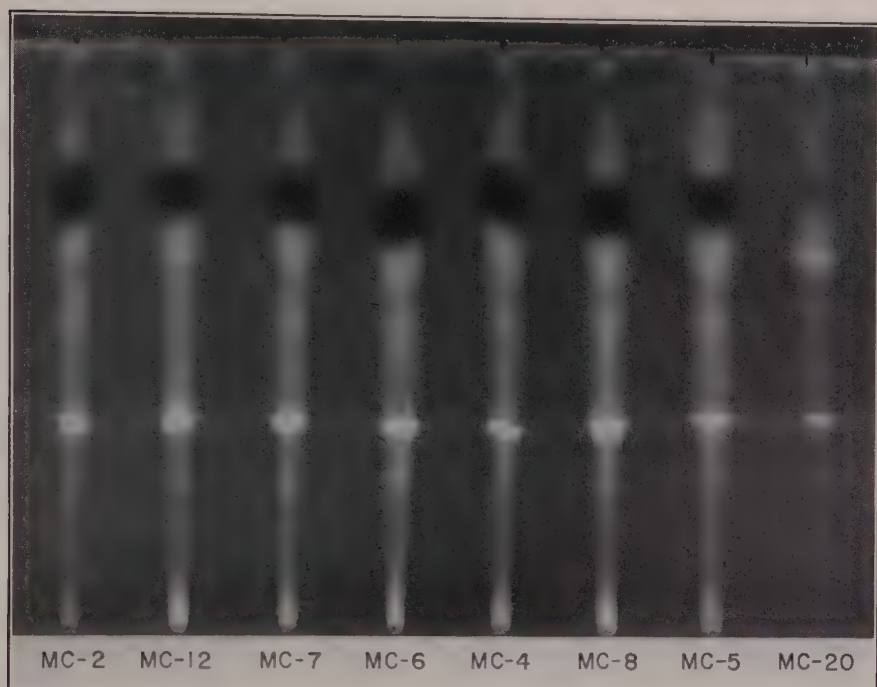


FIGURE 5. Patterns obtained on extracts of Bourbon third beans prepared in different ways (1) and chromatographed with KOH-NaBr-ethanol. MC-2: Extracted 2 hours at  $120^{\circ}$  F. MC-12: Autoclaved for 2.5 hours at 15 p.s.i. MC-7: Extracted for 2 hours at  $173^{\circ}$  F. in presence of  $\text{KHCO}_3$ . MC-6: Extracted for 2 hours at  $173^{\circ}$  F. in presence of  $\text{K}_2\text{CO}_3$ . MC-4: Extracted for 2 hours at  $173^{\circ}$  F. in presence of KOH at a pH of 6.1 to 6.6. MC-8: Extracted with isopropanol in presence of KOH. MC-5: Same as MC-4 except the pH range was 7.1 to 8.4. MC-20: Extract prepared from exhausted beans.

15 lbs. steam pressure or in the presence of  $\text{KHCO}_3$ ,  $\text{K}_2\text{CO}_3$ , and KOH at pH values above and below 7, or with a menstruum containing both KOH and isopropanol. All of the extracts contained the fluorescent components characteristic of authentic vanilla except those prepared from spent beans (Fig. 5). In some of the more severe treatments black materials were produced which streaked on the papers, but, after these settled out during aging of the products, the chromatographic patterns were found to be almost identical with those of extracts prepared by the standard F.E.M.A. method (3). Thus, it is likely that tenfold extracts produced under industrial conditions will have acceptable patterns.

Extracts of other plants also produce chromatographic patterns, but these in no way resemble those obtained on vanilla. Therefore, this method can be used in many cases to detect adulteration with foreign botanicals (Fig. 6). Extracts of hickory bark, cherry bark, angostura, St. John's-wort,

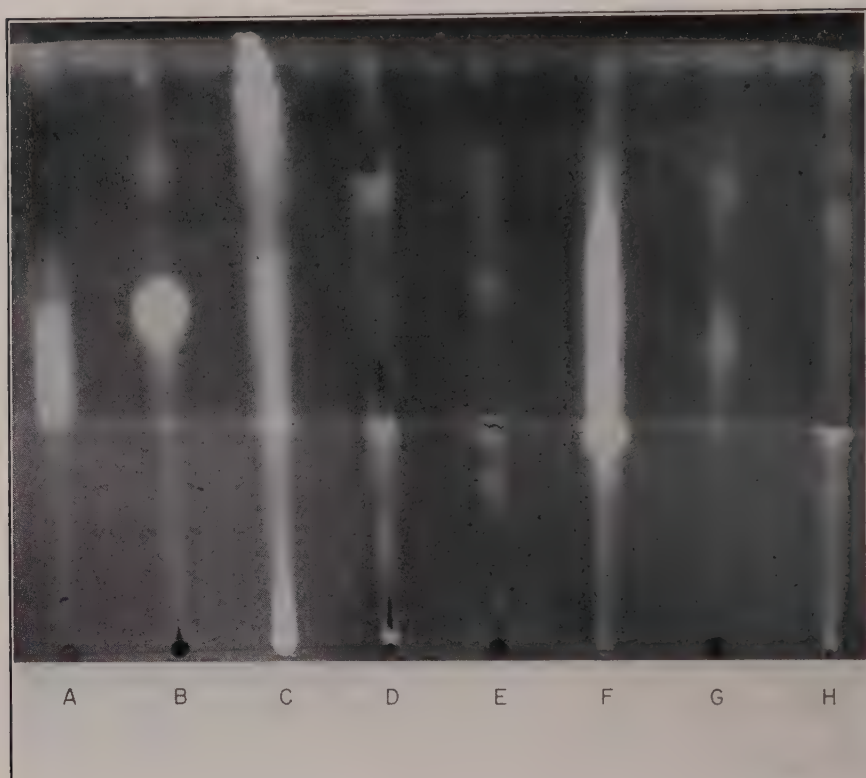


FIGURE 6. Chromatograms obtained with KOH-NaBr-ethanol on plant extracts other than vanilla. A. Hickory bark, B. cherry bark, C. angostura, D. St. John's-wort, E. rhubarb root, F. licorice, G. black walnut, H. cascara.

rhubarb root, licorice, black walnut, and cascara all contain fluorescent compounds, dark materials, and in some cases pigments that are visible under ordinary illumination (Fig. 6). Other plant extracts which are not illustrated but which can be detected in this way include coffee, tea, fenugreek, prune juice, buckthorn, prickly ash, pipsissawa, burdock root, cacao, kola nut, and horehound. Some of these extracts contain components that fluoresce so brilliantly that they can be perceived immediately when present in vanilla, while others are masked by the vanilla pattern. Since the ease with which these materials can be detected depends on the concentration used, no general statement can be made concerning the specific extracts that can be detected when used to adulterate vanilla.

#### RESULTS ON COMMERCIAL VANILLA EXTRACTS

Seven samples of household vanilla extract purchased in various sections of the United States by members of the Flavoring Extract Manufac-

turers' Association and submitted under code were examined by paper chromatography. Four of these (H-2, H-3, H-4, and H-7) were obviously diluted (Fig. 7). In addition, H-4 was probably adulterated since it contained a bright fluorescent component at  $R_f$  0.9 in contrast to the general weakness of the fluorescence elsewhere. Samples H-5 and H-6 gave acceptable vanilla patterns, although the fluorescence is somewhat weaker than that of the standard. The sample coded H-1 evidently contained a large amount of sugar since development of the chromatogram was retarded. Redevelopment in the same solvent did not eliminate this entirely, so it was necessary to extract this sample with chloroform-amine reagent to demonstrate the presence of an authentic, although somewhat weak, vanilla pattern. Thus, of the seven samples examined, one was diluted and probably adulterated, three were definitely diluted, and the remaining three gave patterns that were somewhat weak but acceptable. It would be necessary to resort to quantitative analysis to show whether the full weight of beans was used in preparing these latter extracts. However, there is no doubt that the other four samples were not representative of full-strength authentic vanilla extract. In addition to the samples illustrated in Figure

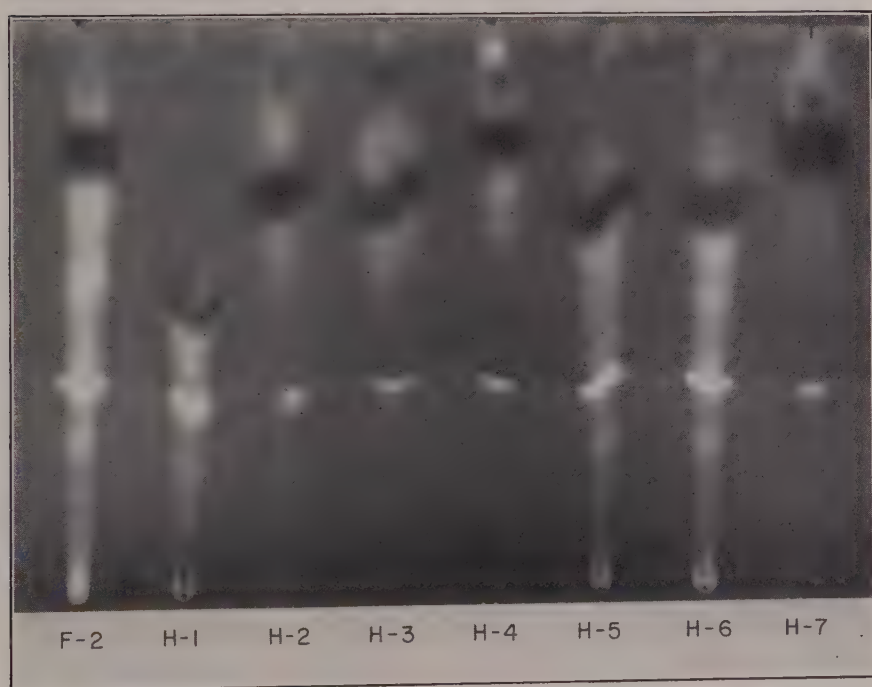


FIGURE 7. Chromatograms obtained on seven commercial household extracts coded H-1 to H-7 compared to authentic Guadeloupe extract (F-2) when developed in KOH-NaBr-ethanol.



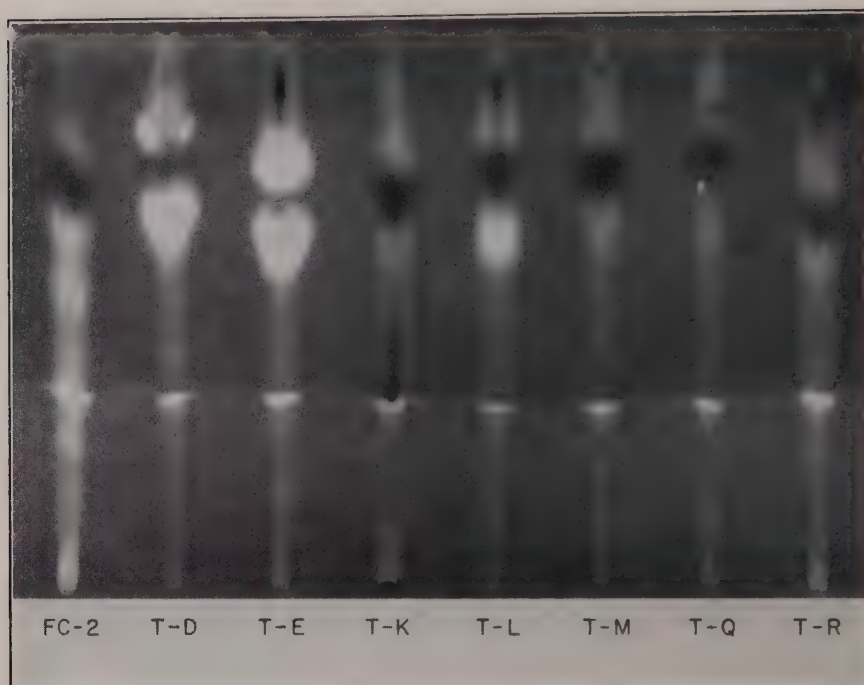


FIGURE 8. Patterns obtained on commercial tenfold extracts (T-series) developed with KOH-NaBr-ethanol compared to a concentrate of Guadeloupe extract (FC-2).

7, a large number of extracts were purchased at chain and independent grocery stores in the New York City area and analyzed. The distribution between acceptable and substandard extracts was about the same as found on the samples submitted by the F.E.M.A. membership.

The quality of commercial tenfold vanilla extracts sold to the dairy and bakery industries was found to be much lower than was the case for the household extracts. Of thirteen samples examined, seven were definitely adulterated. The tenfolds coded T-D, T-E, T-K, and T-L (Fig. 8) are particularly obvious examples of adulteration. They would immediately be classified as spurious even by the most inexperienced observer. The difference between these samples and authentic vanilla extract (FC-2) is obvious even on a black and white reproduction (Fig. 8), and this effect is much enhanced when it is possible to see areas of blue, blue green, green, yellow, and orange fluorescence. T-R is also adulterated although this cannot be detected on a black and white photograph. T-Q is possibly adulterated, has a very weak fluorescent pattern and, in addition, did not contain the volatile components present in authentic vanilla when examined by gas chromatography (1). In summary, seven of these samples were defi-

nately adulterated, four were possibly adulterated, one gave an extremely weak pattern and one (tenfold W) gave an acceptable although weak fluorescent pattern when examined by paper chromatography. It is interesting that this sample also gave an acceptable fractogram when examined by gas chromatography (1).

It is evident that standards of quality in the tenfold industry, and to a lesser extent in the household field, are so low that paper and gas chromatography alone will suffice at present for detecting adulterated and diluted products. However, as the more flagrant violations disappear, it will be necessary to use quantitative procedures to obtain evidence on manufacturers who consistently market products which are 10 to 25 per cent understrength. It is possible that this can be accomplished by methods described elsewhere (5).

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# EFFECTS OF 5-NITROFURAN DERIVATIVES ON ORGANIC ACID METABOLISM OF CONIDIA OF *NEUROSPORA SITOPHILA* AND ON ACETYLATION OF SULFANILAMIDE *IN VITRO*<sup>1</sup>

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## SUMMARY

Nine derivatives of 5-nitrofuran variously substituted in the 2-position were tested for effects on organic acid metabolism of conidia of *Neurospora sitophila*. The compounds were 5-nitro-2-furaldoxime (I), ethyl 3-(5-nitro-2-furyl)acrylate (II), 2,5-dinitrofuran (III), 5-nitro-2-furonitrile (IV), 3-(5-nitro-2-furyl)acrolein (V), 1-(5-nitro-2-furyl)-2-nitroethylene (VI), 1-(5-nitro-2-furyl)-2-nitro-1-propene (VII), 1-nitro-4-(5-nitro-2-furyl)-1,3-butadiene (VIII), and 3-(5-nitro-2-furyl)acrolein oxime (IX). All of the compounds inhibited synthesis of citrate from acetate to some extent. II, IV, V, VII, VIII, and IX inhibited metabolism of pyruvate, and I and II inhibited fumarase activity *in vivo*.

All of the compounds at concentrations of 31  $\mu M$ /l. and above inhibited to some degree acetylation of sulfanilamide by the acetylating system from pigeon liver, with acetate as the acetyl source. The most potent inhibitors were III, V, and VI. I, IV, and Furacin inhibited the system strongly only at the higher concentrations. Evidence is presented that CoA is inactivated to some extent by some of the derivatives but that the major action is on the apoenzymes in the system. The results are discussed with respect to differences in activity of different derivatives and differences in toxicity to fungi and bacteria.

## INTRODUCTION

Following the discovery of 5-nitrofuran derivatives with high antibacterial activity (3), considerable research effort has been directed toward elucidation of their mode of action. Asnis and Gots (1, 2) reported that Furacin (5-nitro-2-furaldehyde semicarbazone) inhibits respiration and pyruvate metabolism of bacteria. Featherstone *et al.* (4) found that chronic feeding of Furadroxyl [5-nitro-2-furaldehyde 2-(2-hydroxyethyl) semicarbazone] to rats results in temporary loss of the capacity of the seminiferous tubules to oxidize pyruvate. Paul *et al.* (10) showed that Furacin inhibits utilization of glucose by brain, testis, diaphragm, and heart tissue slices under aerobic, but not under anaerobic, conditions. Later they showed that formation of citrate from pyruvate and oxalacetate or malate is inhibited *in vitro* (13). Working with partially purified enzymes (13), they found that transfer of the acetyl group to oxalacetate or sulfanilamide was not inhibited appreciably by Furacin at low concentrations

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<sup>2</sup> Research Fellow under terms of a grant from the International Cooperation Administration administered by the National Academy of Sciences.

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with acetyl coenzyme A or with acetate as the acetyl source, whereas formation of acetyl CoA from pyruvate was strongly inhibited under  $N_2$  with pyruvate as the acetyl source. They concluded that Furacin inhibits the anaerobic formation of acetyl coenzyme A from pyruvate but does not inactivate coenzyme A *per se*.

The study reported here was carried out in conjunction with investigations of other aspects of nitrofurantoin action against fungi (7, 9). Various substituted 5-nitrofurantoin derivatives were tested for effects on organic acid metabolism of intact fungus spores *in vivo* and on the acetylation of sulfanilamide *in vitro* by the acetylating enzyme from pigeon liver, with acetate as the acetyl source.

### MATERIALS AND METHODS<sup>3</sup>

Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge were employed for all experiments requiring spores. The fungus was cultured and conidia were harvested and prepared for testing as described previously (6). Results are expressed in terms of dry weight of the spores. Dry weight was determined on 2-ml. aliquots taken from a freshly prepared aqueous spore suspension which served as the stock for experimental samples. The spores were dried for 8 hours at 100° C.

Systems for determining the effects of the toxicants on organic acid metabolism in intact spores were made up as follows: spores, approximately 500 mg. in 20 ml. of water; sodium acetate, 200 mg. in 20 ml. of water; the lethal dose of toxicant in 2 ml. of acetone; and distilled water to make a total volume of 60 ml. Controls contained all components except the toxicant and, when appropriate, acetate. All systems were prepared in duplicate in 250-ml. Erlenmeyer flasks and incubated for 16 hours on a reciprocating shaker. The spores do not germinate until diluted and supplied with biotin. The lethal amount of each toxicant was determined in preliminary experiments by varying the amount of toxicant in the systems described above. The dosage employed is given in Table I. Viability of the spores was determined as described before (8).

Methods for extraction and determination of organic acids were described or cited previously (8). Incubation solutions were combined with spore extracts so that leaching of cell contents was not a factor in the reported results.

Acetylation of sulfanilamide was carried out with the acetylating enzyme from pigeon liver prepared and tested by the method of Kaplan and Lipman (5). The CoA source was a commercial preparation containing 70 to 75 per cent CoA from Nutritional Biochemicals Corporation, Cleveland, Ohio. ATP was chromatographically pure and was obtained from the

<sup>3</sup> Abbreviations used are: CoA, coenzyme A; TCA cycle, tricarboxylic acid cycle; ATP, adenosine triphosphate.

TABLE I

EFFECTS OF VARIOUS NITROFURAN DERIVATIVES AT LETHAL DOSES ON ORGANIC ACID LEVELS IN CONIDIA OF *NEUROSPORA SITOPHILA*

| Chemical                                      | Dosage,<br>mg./g.<br>spores† | Amount of acid, mg./g. spores |                |               |        |                              |               |
|-----------------------------------------------|------------------------------|-------------------------------|----------------|---------------|--------|------------------------------|---------------|
|                                               |                              | Citrate                       | Suc-<br>cinate | Fu-<br>marate | Malate | $\alpha$ -Keto-<br>glutarate | Pyru-<br>vate |
| None—not incubated                            | None                         | 1.7                           | 3.4            | 0             | 1.0    | 0.02                         | 0.02          |
| None—incubated*                               | "                            | 3.0                           | 1.5            | 0             | 1.1    | 0.04                         | 0.01          |
| None—incubated with<br>acetate**              | "                            | 10.5                          | 3.4            | Trace         | 2.1    | 0.10                         | 0.07          |
| 5-Nitro-2-furaldoxime                         | 39.0                         | 4.0                           | 3.0            | 12.5          | 3.3    | 0.01                         | 0.05          |
| Ethyl 3-(5-nitro-2-furyl)-<br>acrylate        | 53.0                         | 4.9                           | 3.8            | 2.8           | 4.4    | 0.08                         | 0.77          |
| 2,5-Dinitrofurane                             | 16.6                         | 2.3                           | 1.6            | Trace         | 0.8    | 0                            | 0             |
| 5-Nitro-2-furonitrile                         | 35.0                         | 2.4                           | 1.6            | "             | 1.1    | 0.07                         | 0.89          |
| 3-(5-Nitro-2-furyl)acro-<br>lein              | 17.6                         | 2.9                           | 1.9            | "             | 2.9    | 0.03                         | 0.31          |
| 1-(5-Nitro-2-furyl)-2-<br>nitroethylene       | 19.3                         | 2.4                           | 1.6            | "             | 1.2    | 0.01                         | 0.06          |
| 1-(5-Nitro-2-furyl)-2-<br>nitro-1-propene     | 43.0                         | 1.4                           | 1.3            | "             | 0.6    | 0                            | 0.09          |
| 1-Nitro-4-(5-nitro-2-<br>furyl)-1,3-butadiene | 27.6                         | 3.7                           | 3.1            | "             | 3.5    | 0.12                         | 0.80          |
| 3-(5-Nitro-2-furyl)acro-<br>lein oxime        | 42.9                         | 2.3                           | 3.0            | "             | 2.6    | 0.01                         | 0.16          |

\* Incubated for 16 hours on a reciprocating shaker.

\*\* The acetate concentration was approximately 400 mg./g. of spores.

† The spores were not viable after incubation with these amounts of the nitrofurans. The toxicant dose is dependent upon the ratio of spores to toxicant. Therefore, the dosages are given in terms of the amount of spores in the system.

same company. Details of the reaction system are given with Table III.

To determine whether the observed inhibition of acetylation resulted from inactivation of CoA or of another component of the system, the CoA and pigeon liver extract were incubated separately for one hour with the toxicant. The toxicant was then removed from the solution by extraction with peroxide-free ether. After extraction, pretreated CoA was mixed with untreated enzyme and they were incubated with other components of the system for 2 hours at 29° C. In other samples the opposite combination was tested, i.e., untreated CoA was mixed with pretreated enzyme.

The nitrofurane derivatives were supplied in crystalline form by The Norwich Pharmacal Company.

## RESULTS AND DISCUSSION

Data in the first three lines of Table I show the amounts of pyruvate and the measurable TCA cycle acids in *N. sitophila* spores at the time of harvest, after incubation for 16 hours in water, and after the same period of incubation with 400 mg. of sodium acetate per gram of spores. The detectable acids were succinate, citrate, malate,  $\alpha$ -ketoglutarate, and pyru-

vate. Glyoxylate was present in small amounts, but since its precursors and metabolic fate in spores are unknown, it is omitted from the present discussion.

Incubation of the spores without a supplemental carbon source resulted in small increases in citrate and  $\alpha$ -ketoglutarate, small decreases in succinate and pyruvate, and no change in malate concentrations. When the incubation medium contained an excess of acetate, all acids except fumarate increased substantially (Table I).

Changes in amounts of TCA cycle acids and pyruvate in the spores following treatment with various 5-nitrofuran derivatives are shown in Table I. Citrate was diminished at least 50 per cent by all derivatives. Accumulation of fumarate was induced by 5-nitro-2-furaldoxime and ethyl 3-(5-nitro-2-furyl)acrylate. Metabolism of pyruvate was blocked to a considerable extent by ethyl 3-(5-nitro-2-furyl)acrylate, 5-nitro-2-furonitrile, 3-(5-nitro-2-furyl)acrolein, 1-nitro-4-(5-nitro-2-furyl)-1,3-butadiene, and 3-(5-nitro-2-furyl)acrolein oxime. It was shown previously that *N. sitophila* spores contain an active pyruvate carboxylase system (6). Accumulation of pyruvate despite the presence of the nonoxidative carboxylase system suggests that cocarboxylase and/or apocarboxylase was inhibited by the above derivatives. Inactivation of cocarboxylase would preclude acetyl-CoA formation from pyruvate under aerobic and anaerobic conditions since the same coenzyme is required under both conditions.

The phenomenon of increase or decrease of specific substrates has been made use of for many years in studies on biochemical genetics where enzyme deficiencies are induced by mutations. Enzyme deficiencies produced by toxicant interaction are equally useful as indications of the sites of toxicant action. The probability of side reactions leading to misinterpretations is reduced to a minimum in the present experiments by simultaneous measurement of all detectable components in a small area of metabolism. Reduced citrate accumulation, induced by all compounds tested, suggests that at least one step in the pathway from acetate to citrate was blocked, or that degradation of citrate was accelerated. The latter interpretation, which assumes uninhibited formation of citrate, requires an increase in at least one other intermediate in the TCA cycle. This requirement exists because the maximum dissimilation rate for each acid was already exceeded by the rate of formation under the conditions of the experiment. This is evident from the net increase in all acids under pressure of excess acetate. Any further increase in the rate of formation of an individual acid would be reflected by a further proportional increase in accumulation of that intermediate.

Ethyl 3-(5-nitro-2-furyl)acrylate and 5-nitro-2-furaldoxime induced substantial increases in fumarate. The latter also increased the total or-

TABLE II

EFFECTS OF 5-NITRO-2-FURALDOXIME AND ETHYL 3-(5-NITRO-2-FURYL)ACRYLATE ON ORGANIC ACID LEVELS IN SPORES OF *N. SITOPHILA* IN THE PRESENCE AND ABSENCE OF EXOGENOUS ACETATE\*

| Nitrofuran derivative             | Dosage, mg./g. spores | Acetate, mg./g. spores | Amount of acid, mg./g. of spores |           |          |        |                         |          |       |
|-----------------------------------|-----------------------|------------------------|----------------------------------|-----------|----------|--------|-------------------------|----------|-------|
|                                   |                       |                        | Citrate                          | Succinate | Fumarate | Malate | $\alpha$ -Ketoglutarate | Pyruvate | Total |
| None                              | None                  | 0                      | 3.0                              | 1.5       | 0        | 1.1    | 0.04                    | 0.01     | 5.65  |
|                                   |                       | 400                    | 10.5                             | 3.4       | Trace    | 2.1    | 0.10                    | 0.07     | 16.17 |
| 5-Nitro-2-furaldoxime             | 39.0                  | 0                      | 3.4                              | 4.3       | 8.3      | 1.6    | 0.01                    | 0        | 17.61 |
|                                   |                       | 400                    | 4.0                              | 3.0       | 12.5     | 3.3    | 0.01                    | 0.05     | 22.86 |
| Ethyl 3-(5-nitro-2-furyl)acrylate | 53.0                  | 0                      | 3.0                              | 4.2       | 2.0      | 1.6    | 0.06                    | 0.12     | 10.98 |
|                                   |                       | 400                    | 4.9                              | 3.8       | 2.8      | 4.4    | 0.08                    | 0.77     | 16.75 |

\* All systems were incubated for 16 hours on a reciprocating shaker.

ganic acid content of the spores (Table II). Superficially, this suggests accelerated assimilation of acetate although citrate did not accumulate as it did in the controls. However, further experiments in which spores were treated with these compounds without addition of acetate show that some TCA cycle intermediates accumulated to abnormally high levels even in the absence of acetate (Table II). The difference between total acids formed from endogenous sources and from acetate was only about 5 mg. in the poisoned spores. In nontreated spores more than 10 mg. of acids could be attributed to assimilation of acetate. Thus, acetate assimilation was inhibited about 50 per cent by the toxicants.

Previous studies (8) have shown that TCA cycle intermediates are formed from dissimilation of endogenous protein, lipids, and amino acids upon prolonged incubation, as in the present experiments. As shown by the second line of Table I, the intermediates do not accumulate appreciably in normal spores. Their accumulation in the presence of 5-nitro-2-furaldoxime and ethyl 3-(5-nitro-2-furyl)acrylate indicates inhibition of fumarase and suggests retardation of other processes which normally lead to complete degradation of endogenous substrates.

#### EFFECTS OF 5-NITROFURAN DERIVATIVES ON ACETYLATION OF SULFANILAMIDE IN VITRO BY THE ACETYLATED ENZYME FROM PIGEON LIVER

The foregoing data indicate inhibition of fumarase by 5-nitro-2-furaldoxime and ethyl 3-(5-nitro-2-furyl)acrylate and inhibition of pyruvate metabolism by ethyl 3-(5-nitro-2-furyl)acrylate, 5-nitro-2-furonitrile, 3-(5-nitro-2-furyl)acrolein, 1-nitro-4-(5-nitro-2-furyl)-1,3-butadiene, and 3-(5-nitro-2-furyl)acrolein oxime. However, they do not indicate the site



of inhibition in the pathway between acetate and citrate where at least two enzymes, e.g., acetic thiokinase and citrogenase, are required along with ATP, CoA, and  $Mg^{++}$ . Effects of the nitrofurans on this system were investigated *in vitro* using the acetylating enzyme from pigeon liver (5) and acetate as the acetyl source. The results are given in Table III.

All of the toxicants at concentrations of  $31 \mu M/l.$  and above inhibited acetylation to some degree. The most potent inhibitors were 1-(5-nitro-2-furyl)-2-nitroethylene, 3-(5-nitro-2-furyl)acrolein, and 2,5-dinitrofuran. 5-Nitro-2-furaldoxime, 5-nitro-2-furaldehyde semicarbazone, and 5-nitro-

TABLE III  
EFFECTS OF NITROFURAN DERIVATIVES ON ACETYLATION BY THE  
ACETYLATING SYSTEM FROM PIGEON LIVER IN VITRO\*

| Nitrofuran derivative                          | Sulfanilamide acetylated, $\mu g.$ , at<br>chemical concentration of, $\mu M/l.$ |      |      |      | Inhibition, per<br>cent, at chemical<br>concentration of,<br>$\mu M/l.$ |     |     |
|------------------------------------------------|----------------------------------------------------------------------------------|------|------|------|-------------------------------------------------------------------------|-----|-----|
|                                                | 0                                                                                | 31   | 157  | 628  | 31                                                                      | 157 | 628 |
| 5-Nitro-2-furaldoxime                          | 40.0                                                                             | 35.1 | 23.4 | 10.8 | 12                                                                      | 41  | 73  |
| 5-Nitro-2-furaldehyde semicarbazone            | 40.0                                                                             | 33.8 | 21.3 | 9.1  | 15                                                                      | 47  | 77  |
| Ethyl 3-(5-nitro-2-furyl)acrylate              | 40.0                                                                             | 33.0 | 17.3 | 6.6  | 17                                                                      | 57  | 84  |
| 2,5-Dinitrofuran                               | 35.5                                                                             | 28.5 | 13.8 | 3.7  | 20                                                                      | 61  | 90  |
| 5-Nitro-2-furonitrile                          | 35.5                                                                             | 33.6 | 23.4 | 11.2 | 5                                                                       | 34  | 68  |
| 3-(5-Nitro-2-furyl)acrolein                    | 35.5                                                                             | 26.1 | 11.6 | 3.6  | 26                                                                      | 67  | 90  |
| 1-(5-Nitro-2-furyl)-2-nitroethylene            | 29.8                                                                             | 17.0 | 7.5  | 2.3  | 43                                                                      | 75  | 92  |
| 1-(5-Nitro-2-furyl)-2-nitro-1-propene          | 29.8                                                                             | 23.0 | 15.7 | 7.0  | 23                                                                      | 47  | 77  |
| 1-Nitro-4-(5-nitro-2-furyl)-1,3-buta-<br>diene | 29.8                                                                             | 21.3 | 13.1 | 4.9  | 29                                                                      | 56  | 84  |
| 5-(5-Nitro-2-furyl)acrolein oxime              | 37.2                                                                             | 28.2 | 15.4 | 9.2  | 24                                                                      | 59  | 75  |

\* The systems were as follows:  $0.39 \mu M$  of sulfanilamide;  $24.5 \mu M$  of sodium acetate;  $3.9 \mu M$  of  $Na_2ATP$ ;  $19.7 \mu M$  of Na citrate;  $80 \mu M$  of  $NaHCO_3$ ;  $10 \mu M$  of cysteine hydrochloride;  $0.25$  ml. of aged enzyme solution. These were added in  $0.7$  ml. of water to  $0.7$  ml. of solution containing  $3.25$  units of CoA and the desired amount of each nitrofuran derivative to make a total volume of  $1.4$  ml. The CoA and nitrofurans were incubated together for 1 hour prior to mixing with other components. Incubation 2 hours in air at  $29^\circ C.$  for the acetylation reaction.

2-furonitrile exhibited strong suppression of acetylation only at the higher concentrations. These findings are in accord with the apparent inhibition of citrate formation shown by the data in Table I.

In order to determine whether inhibition of acetylation was due to inactivation of CoA or some other component of the system, solutions of CoA and the enzyme were incubated separately for one hour with inhibitory amounts of 1-(5-nitro-2-furyl)-2-nitroethylene, 1-(5-nitro-2-furyl)-2-nitro-1-propene, or 3-(5-nitro-2-furyl)acrolein. After incubation, the toxicants were removed by extraction with ether. The CoA solution preincubated with the toxicant was mixed with untreated enzyme solution and other components of the reaction mixture and incubated for 2 hours at  $29^\circ C.$  in a water bath. Similarly, the enzyme solution preincubated with

toxicant was combined with untreated CoA solution and the reaction was carried out as before. The results of these treatments are shown in Table IV.

Extraction with ether was found to suppress acetylation to a considerable extent. However, further suppression occurred when either the CoA or enzyme solution was preincubated with the nitrofuran. Effects on CoA were small compared with those on the enzymes, except in the case of 3-(5-nitro-2-furyl)acrolein. When the CoA solution was extracted with ether about 60 per cent of the CoA was apparently inactivated by 3-(5-

TABLE IV

EFFECTS OF THREE NITROFURAN DERIVATIVES ON THE COENZYME A AND APOENZYME FRACTIONS OF THE ACETYLATING SYSTEM FROM PIGEON LIVER IN VITRO\*

| 5-Nitrofuran derivative,<br>0.22 $\mu M$ | Fraction preincubated<br>with chemical (1 hr.) | Inhibition, per cent    |                       |
|------------------------------------------|------------------------------------------------|-------------------------|-----------------------|
|                                          |                                                | Chemical<br>not removed | Chemical<br>removed** |
| 1-(5-Nitro-2-furyl)-2-nitro-1-propene    | Apoenzyme                                      | 73                      | 52                    |
|                                          | Coenzyme A                                     | 15                      | 13                    |
| 1-(5-Nitro-2-furyl)-2-nitroethylene      | Apoenzyme                                      | 79                      | 72                    |
|                                          | Coenzyme A                                     | 28                      | 36                    |
| 3-(5-Nitro-2-furyl)acrolein              | Apoenzyme                                      | 75                      | 50                    |
|                                          | Coenzyme A                                     | 19                      | 63                    |

\* The complete reaction system is given with Table III. Inhibition values are the average from 2 experiments.

\*\* Chemical removed by extraction with ether. Less than 0.0022  $\mu M$  of chemical remained in the solution after extraction. This amount has no effect on the enzyme.

nitro-2-furyl)acrolein. When the chemical was not removed apparent inactivation was only 19 per cent. The reason for this is not known. A check of absorbance of the ether extract at 260 m $\mu$  revealed that no appreciable CoA was extracted whereas most of the nitrofuran was removed. It is concluded that some nitrofurans at high concentrations inactivate CoA to some extent but that the major action is on the apoenzymes in the system. This is in general accord with the conclusions of Paul *et al.* (12, 13) who showed that certain nitrofurans at low concentrations do not inactivate CoA *per se*. They also found that Furacin did not inhibit acetylation of sulfanilamide with acetate as the acetyl source. The data in the present study (Table III), however, indicate that Furacin is inhibitory. The anomaly presented by these data may have derived from the fact that CoA and Furacin were incubated together for 1 hour before mixing with other components in the present experiments. The data show also that some other derivatives have a powerful inhibitory effect on acetylation when present in high concentrations. They show further that different derivatives not

only vary with regard to their potency but sometimes affect different processes so that although the compounds are related by virtue of the 5-nitro group and the furan ring, the 2-substituent might be the key factor in fungicidal activity. The fact that the 2-substituents of many derivatives are unrelated makes it impossible to generalize about the mechanism of action of the nitrofurans as a group.

Paul *et al.* (12) found that the formation of citrate *in vitro* with pyruvate as the acetyl source was inhibited by very low concentrations (0.3 mg./l) of Furacin under anaerobic, but not under aerobic, conditions. While anaerobic metabolism of some tissues and organisms is vital for growth. *N. sitophila* spores germinate only under aerobic conditions and they do not produce CO<sub>2</sub> from sugars under anaerobic conditions. Thus, fungitoxicity must be elucidated in the presence of oxygen, which probably accounts in part for the high doses of nitrofurans required to kill the spores. The present *in vivo* data (Table I) show some of the effects produced by the toxicants when the spores are killed. Since the effects on metabolism and on germination are mutually related to toxicant concentration it seems logical to conclude that interference in metabolism of the spores at the sites pointed out above are in part responsible for the failure of the spores to germinate. Interference with chemically similar enzymes at other sites in metabolism probably also contribute to the over-all toxic mechanism.

A comparison of the present data with those of others obtained with other organisms and animal tissues (1, 2, 4, 10, 11, 12, 13) points to a possible fundamental difference in the mechanism of action of certain nitrofurans in different types of organisms. This is perhaps not surprising in view of the fact that the most potent nitrofurans against bacteria are relatively poor fungicides and that the most potent commercial fungicides are usually not very effective against bacteria. The present data show that certain nitrofurans inactivate processes other than or in addition to pyruvate metabolism, elucidated previously (12, 13). Unfortunately, too little information is available at present to permit quantitative evaluation of the relative importance of inhibition of pyruvate metabolism as compared with inhibition of other processes, such as citrate formation from acetate and fumarate metabolism, in over-all chemical toxicity.

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## THE HISTOPATHOLOGICAL EFFECTS OF OZONE ON PLANT FOLIAGE

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### SUMMARY

Young plants of 32 species and varieties that were growing vigorously in the greenhouse were exposed to ozone at concentrations of 0.1 to 1.0 p.p.m. in a circulating stream of washed air. In descending order of susceptibility the plants were tomato, bean (Pinto), tobacco (Maryland Mammoth), spinach, potato, smartweed, stevia, tobacco (Vamorr 48), chrysanthemum (Pippin), hypericum, alfalfa, peanut, tobacco (Turkish), sweet potato, sensitive plant, grape, verbenia, chrysanthemum, Jerusalem cherry, avocado, sugar-beet, strawberry, mint, and geranium. Under the conditions of this test, gladiolus, kalanchoe, pepper, bean (Tendergreen), and piggy-back plant were not visibly injured by concentrations up to 1.0 p.p.m. Foliage injury appeared in varying amounts as dark stipples, light flecks, necrotic patches, and general chlorosis.

The dark punctate markings were shown to be due to pigmentation and thickening of cell walls in the palisade area. The light-colored flecks were associated with a collapse of the palisade cells without pigment formation. The necrotic areas were found to consist of general injury to all cells between and including the epidermis in the affected area.

In general, very young leaves that had no functional stomata were resistant to ozone. The leaves become susceptible only as the stomata become functional. In variegated coleus injury was largely associated with the pigmented sectors presumably due to the greater functioning and frequency of the stomata, or to the greater sensitivity of these tissues to ozone.

### INTRODUCTION

Ozone toxicity is a recognized problem in the Los Angeles, California, area and is of general interest wherever atmospheric mixtures of ozone and hydrocarbons occur (1, 3, 4, 11, 16, 17, 18). The effects of ozone on lemon fruit respiration (19), mitochondrial oxygen uptake (6), and photosynthesis and chlorophyll content (5) have been reported. Foliar effects of ozone on peas, Brussels sprouts, wheat, and rye have been described as bleaching (10) and on bean seedlings as bleaching and spotting (8, 13). Middleton and Paulus (14, Figs. 3-6) show by a color plate the effects of ozone on bean seedling leaves compared to the effects of sulfur dioxide, and the effects of smog or oxidized hydrocarbon on *Chenopodium*, romaine lettuce, and annual bluegrass. The extent of foliar bleaching as a function of ozone concentration and exposure time has been reported (12, Figs. 4, 5). The present study was made to determine the gross pattern of injury, the relative susceptibility, and histological effects on a number of plant species to ozone fumigation.

<sup>1</sup> Deceased August 14, 1958.

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## MATERIALS AND METHODS

Controlled concentrations of ozone were produced in a fumigating cabinet from a Welsbach Model T-23 Laboratory Ozonator. Concentrations from less than 0.1 part per million (p.p.m.) by volume to over 4 p.p.m. were obtained in the cabinet. Oxygen was used for ozone production in most experiments to avoid the production of nitrogen oxides. However, the markings on the plants appeared the same when dry air was used as a source.

The fumigating cabinet was similar to that described previously (22, pp. 264-266), except that it was located in a greenhouse and its capacity was 200 cubic feet. Ozone was injected into the ingressing stream of scrubbed air for 4 or 8 hours during daylight. The air flow was maintained at a sufficient rate for complete exchange approximately once per minute.

The concentration of ozone in the cabinet was determined by drawing a measured air sample through a basic potassium iodide solution contained in two bubblers connected in series. The iodine, released after acidification, was titrated with 0.01*N* sodium thiosulfate solution, using starch solution as an indicator (9, pp. 292-293). Samples of the ambient air showed an undetectable oxidant content by this method.

## RESULTS AND DISCUSSION

*Types of markings produced by ozone fumigation.* The species listed in Table I were held in the fumigating cabinet for 8 hours at concentrations from 0.1 to 1.0 p.p.m. ozone. The four types of markings which were produced on these plants were: 1) small dark stipples, 2) light fleck-like markings, 3) necrotic patches, and 4) yellowing of the entire leaf. The ozone markings were not exclusively of one type or another since intermediate forms also were found.

Dark punctate markings were consistently produced on leaves of grape (vars. Zing, Tokay and Concord), avocado, and frequently on chrysanthemum and strawberry; they were produced with less consistency on leaves of coleus, sweet potato, and mint, and only occasionally on Pinto bean leaves. These markings have been described by Richards, Middleton, and Hewitt (15) as "oxidant stipple." The stipples were easily seen on the upper surface of the leaf as in the avocado (Fig. 1 A), and resulted from thickening and pigmentation of the walls in groups of palisade cells (Fig. 1 B, C). The overlying epidermal and surrounding mesophyll cells appeared to be unaffected. There was a sharp demarcation, with no gradation, between affected and adjacent unaffected cells.

On the leaves of some plants such as sugar-beet (Fig. 2 A), smartweed (Fig. 3), and tobacco, light-colored fleck-like patterns were produced which were larger than the stipple of grape and remained light in color. Peanut and sweet potato occasionally showed this type of marking.

TABLE I

RELATIVE SUSCEPTIBILITY OF PLANTS EXPOSED FOR EIGHT HOURS TO OZONE. THE SPECIES ARE ARRANGED IN DECREASING ORDER OF SUSCEPTIBILITY BASED ON THE APPROXIMATE THRESHOLD CONCENTRATION FOR MARKING OF FOLIAGE

| Species                                                          | Suscep-<br>tibility* | Type of marking** |         |       |
|------------------------------------------------------------------|----------------------|-------------------|---------|-------|
|                                                                  |                      | Necrotic<br>patch | Stipple | Fleck |
| Tomato, <i>Lycopersicon esculentum</i> Mill. var. Bonny Best     | XXX                  | ++                |         |       |
| Bean, <i>Phaseolus vulgaris</i> L. var. Pinto                    | XXX                  | +                 | +       |       |
| Tobacco, <i>Nicotiana tabacum</i> L. var. Maryland Mammoth       | XXX                  | ++                |         |       |
| Spinach, <i>Spinacia oleracea</i> L. var. Early Hybrid 7         | XXX                  | ++                |         |       |
| Potato, <i>Solanum tuberosum</i> L.                              | XXX                  | ++                |         |       |
| Smartweed, perennial, <i>Polygonum</i> sp.                       | XXX                  |                   |         | ++    |
| Stevia, <i>Piqueria trinervia</i> Cav.                           | XX                   | ++                |         |       |
| Tobacco, <i>Nicotiana tabacum</i> L. var. Vamorr 48              | XX                   |                   |         | ++    |
| Coleus, <i>Coleus blumei</i> Benth.                              | XX                   | +                 | +       |       |
| Chrysanthemum, <i>Chrysanthemum</i> sp. var. Pippin              | XX                   | +                 | +       |       |
| Hypericum, <i>Hypericum</i> sp.                                  | XX                   | ++                |         |       |
| Alfalfa, <i>Medicago sativa</i> L.                               | XX                   | ++                |         |       |
| Peanut, <i>Arachis hypogaea</i> L.                               | XX                   | +                 |         | +     |
| Tobacco, <i>Nicotiana tabacum</i> L. var. Turkish                | XX                   | +                 |         | ++    |
| Sweet potato, <i>Ipomoea batatas</i> Lam. var. Porto Rico        | X                    | +                 |         | +     |
| Sensitive plant, <i>Mimosa pudica</i> L.                         | X                    | ++                |         |       |
| Grape, <i>Vitis</i> sp. vars. Zing, Tokay, Concord               | X                    |                   | ++      |       |
| Verbena, <i>Verbena</i> sp. var. L. A.                           | X                    | ++                |         |       |
| Chrysanthemum, <i>Chrysanthemum</i> sp.                          | X                    |                   | ++      |       |
| Jerusalem cherry, <i>Solanum pseudo-capsicum</i> L.              | X                    | +                 |         |       |
| Avocado, <i>Persea</i> sp.                                       | X                    |                   | ++      |       |
| Sugar-beet, <i>Beta vulgaris</i> L.                              | X                    | +                 |         | ++    |
| Strawberry, <i>Fragaria</i> sp. var. Empire                      | X                    |                   | ++      |       |
| Mint, <i>Mentha piperita</i> L.                                  | X                    |                   | +       |       |
| Geranium, <i>Geranium</i> sp.                                    | X                    | ++                |         |       |
| Gladiolus, <i>Gladiolus</i> sp. var. Snow Princess               | O                    |                   |         |       |
| Kalanchoe, <i>Kalanchoe diargemontiana</i> Hamet & Perrier       | O                    |                   |         |       |
| Pepper, <i>Capsicum frutescens</i> L. var. <i>grossum</i> Bailey | O                    |                   |         |       |
| Bean, <i>Phaseolus vulgaris</i> L. var. Tendergreen              | O                    |                   |         |       |
| Piggy-back plant, <i>Tolmiea menziesii</i> Torr. & Gray          | O                    |                   |         |       |

\* XXX=Marked at less than 0.1 p.p.m. ozone; XX=marked at 0.1 to 0.3 p.p.m. ozone; X=marked at 0.3 to 1.0 p.p.m. ozone; O=not marked at 1.0 p.p.m. ozone.

\*\* ++=Marking consistently formed; +=marking occasionally formed.

Heggstad and Middleton (7) have found good correlation in the field between "weather fleck" of tobacco and ozone concentration in the air. Microscopic examination revealed that groups of palisade cells in the light areas collapsed and became bleached. The affected cells readily stained with a weak aqueous thionine solution which Bobrov (2) found especially useful in detecting early cell damage from smog.

On more tender leaves such as those of tomato, potato, and *Hypericum* (Fig. 2 B), necrotic areas were produced which extended from surface to surface through the leaf. These areas, which were usually intercostal and intermarginal, varied in diameter from 1 to 10 mm. With this type of marking all the cells between and including the epidermal layers were injured, and the resulting papery area usually became light tan in color or



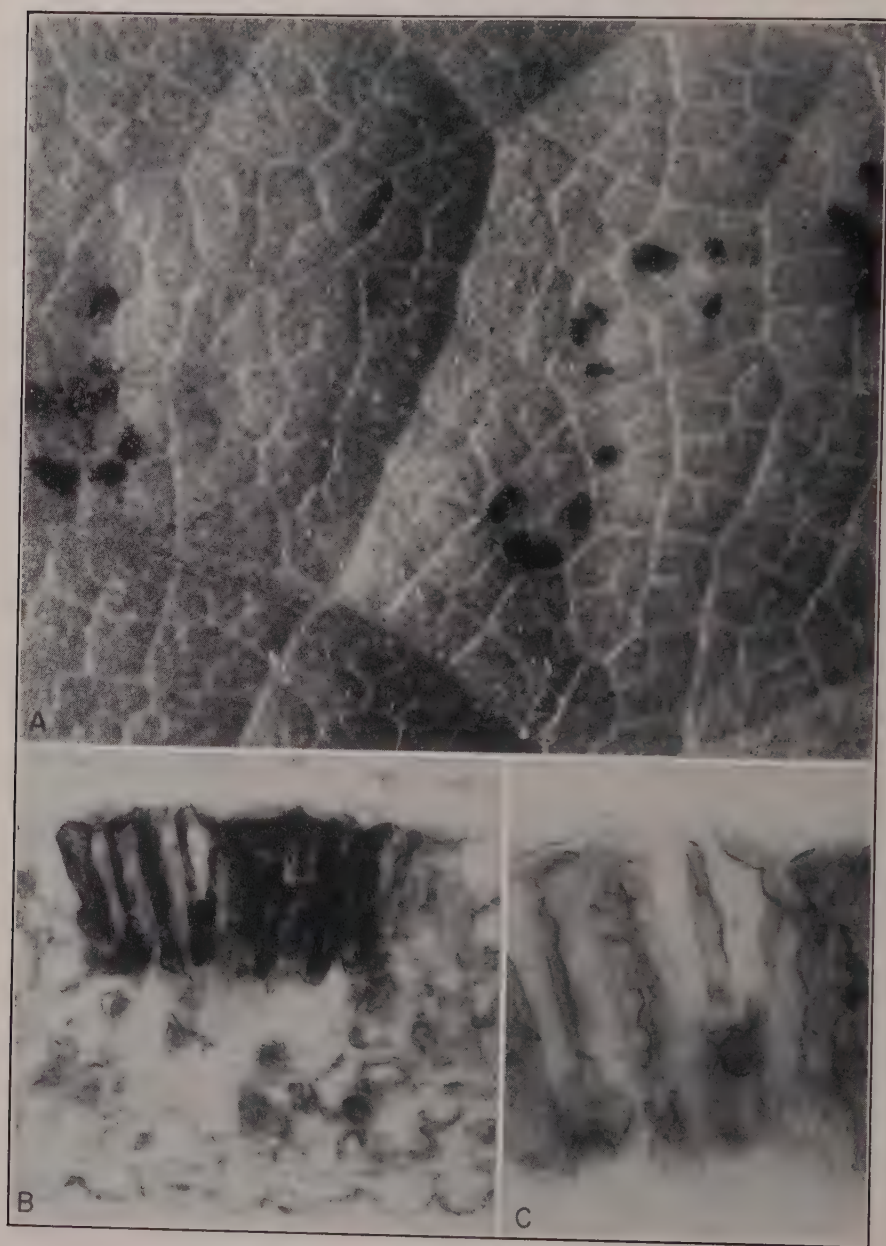


FIGURE 1. Stipple marks on avocado leaf photographed one week after an 8-hour fumigation with 0.5 p.p.m. ozone. A. View of upper surface ( $\times 18$ ). B. Fresh cross section of same leaf with darkened palisade cells ( $\times 380$ ). C. Details of palisade cells in B showing thickened walls ( $\times 870$ ).



FIGURE 2. A. Sugar-beet leaves of increasing age (left to right) photographed from upper surface 4 days after an 8-hour fumigation with 0.7 p.p.m. ozone ( $\times 0.58$ ). B. Successive *Hypericum* leaves of increasing age (left to right) photographed 2 weeks after an 8-hour fumigation with 0.2 p.p.m. ozone showing light-colored areas of injury from lower surface ( $\times 0.34$ ).



FIGURE 3. Smartweed leaves of increasing age (left to right) photographed from upper surface 4 days after 4-hour fumigation with 0.25 p.p.m. ozone ( $\times 0.72$ ).

sometimes reddish as in *Hypericum*. In the older leaves of sugar-beet, peanut, and stevia, the affected areas were occasionally marginal. The oldest beet leaf (Fig. 2 A, at right) became dried and curled along most of the margin.

The older leaves of some plants such as tomato, coleus, and sweet potato lost their chlorophyll and became yellow. Such leaves usually abscised prematurely.

*Effect of tissue maturity.* In some plants specific zones of the leaf showed ozone injury depending on the age of the leaf. Such a pattern was seen on the leaves of *Hypericum* (Fig. 2 B), the younger leaves of which showed a sensitive zone at the tip which progressed to the base of the older leaves. This follows the same progression of maturity and oxidant sensitivity that Bobrov (2) reported for *Poa annua*. Very young leaves, with nonfunctional stomata, were resistant to ozone regardless of the plant species tested. As the leaf tissues matured and the stomata became functional the leaves became susceptible to ozone; later the more mature leaf tissue became resistant to ozone. Evidently *Hypericum* falls into the class of narrow-leaved dicotyledons described by Ziegenspeck (21) which differentiate their stomata basipetally beginning at the tip of the leaf. As senescence was approached the leaves of some species such as tomato, coleus, and sweet potato again became ozone-sensitive and turned yellow. At this stage the stomata probably cease to function and remain open.

*Effect of ozone on variegated leaves.* When a green and white variegated variety of coleus was fumigated with 0.1 p.p.m. ozone, the injury was confined almost exclusively to the green portions of the leaf. This may be due to one or more of the following factors: an effect on the photosynthetic mechanism (5); the greater responsiveness of stomata to light over green areas (20); of the greater number of stomata over the green area than the white area. The numbers of stomata per square millimeter in the upper epidermis were 25 for the white areas and 28 for the green areas. For the lower epidermis the corresponding numbers were 151 for the white areas and 302 for the green areas.

*Relative sensitivity of various species of plants to ozone.* Table I shows the species tested, their susceptibility to ozone, and types of markings produced. Species which consistently show stippling have relatively coreaceous leaves and are sensitive to only the relatively high concentrations of ozone (0.3 to 1.0 p.p.m.).

#### ACKNOWLEDGMENT

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# GROWTH SUBSTANCES FROM MARYLAND MAMMOTH TOBACCO: LONG CHAIN ALCOHOLS

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## SUMMARY

The extraordinary growth properties of Maryland Mammoth tobacco, *Nicotiana tabacum* L. var. Maryland Mammoth, and the apparent absence of indole-3-acetic acid, prompted further investigation of the factors responsible for growth in this variety. A growth-promoting fraction was isolated from an alcoholic extract of leaves and apical tissue by means of paper chromatography and was shown to have properties characteristic of long-chain, primary alcohols containing between 22 and 28 carbon atoms. Bioassay of a wide variety of long-chain compounds by the oat first-internode method of Nitsch and Nitsch revealed that a number of C<sub>17</sub> to C<sub>22</sub> alcohols and their acidic inorganic esters exhibited growth-promoting activity and that structural requirements for activity were quite specific for these alcohols. This finding suggests that present concepts of the hormonal regulation of plant cell extension may be inadequate and should be re-evaluated.

## INTRODUCTION

Maryland Mammoth tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth) possesses several characteristics which differentiate it from most other varieties of the species. The most conspicuous are its very vigorous growth habit and large leaf size compared with other types of tobacco, and its dependence upon a short-day photoperiod for flowering. These features suggested that an unusual type of hormonal growth control might be in operation. Results of previous work (9) supported this hypothesis, and, in fact, the presence of the classical auxin, indole-3-acetic acid (IAA), could not be demonstrated by Vlitos *et al.* (8, 9) in this plant. Consequently, further attempts were made to define more clearly the chemical factors responsible for its growth and development.

Several growth-promoting substances were isolated from extracts of tobacco by means of paper chromatography. Their identification is of great importance to the understanding of natural growth regulation in this species in the absence of the auxin system based on IAA. Studies were directed toward the purification and structure determination of the Maryland Mammoth growth substances and the eventual physiological comparison of their homologues and other closely related compounds. Evidence is presented in this paper that one growth promoter has the chemical characteristics of a long-chain fatty alcohol, and that this type of biological activity is present in only a few of the long-chain compounds which were bioassayed.

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MATERIALS AND METHODS<sup>3</sup>

## ISOLATION OF A GROWTH-PROMOTING FRACTION

Leaves and apical tissue of two-month-old Maryland Mammoth tobacco plants (500 grams) were harvested, frozen in solid carbon dioxide, and ground in absolute ethanol. The initial grinding was made in a Waring Blendor, followed by homogenation in a VirTis H-5 homogenizer. The alcoholic homogenate was allowed to stand 14 hours at  $-4^{\circ}\text{C}.$ , centrifuged free of plant debris at  $-10^{\circ}\text{C}.$ , and the supernatant was concentrated under reduced pressure. The clear, brown, aqueous solution thus obtained was acidified to pH 3 with 0.1*N* phosphoric acid, extracted with three portions of diethyl ether, and the extracts were combined and concentrated.

The concentrate was introduced into the top of a Grycksbo chromatographic filter paper column (Type LKD-3391),<sup>4</sup> and was followed by a steady flow of a developing solvent consisting of isopropyl alcohol, concentrated ammonium hydroxide, and water, in the proportions of 80:5:15 by volume. Successive 100-ml. fractions of percolate were removed at the bottom of the column until a total of 3 l. had been collected, and each fraction was stored in the cold.

After several days, it was observed that a white, oily precipitate had separated in fractions 25 to 28. This material was isolated by centrifugation and dried over anhydrous calcium chloride. The dry, brown solid was warmed with a few ml. of absolute ether in a centrifuge cone, the mixture was centrifuged, the supernatant was transferred to a clean cone, and its volume was reduced to about 1 ml. under a stream of dry nitrogen. After storage at  $-20^{\circ}\text{C}.$  for several days, the precipitated white crystals were centrifuged in an International refrigerated centrifuge. The supernatant was then removed, and the solid was mixed again with warm ether. This procedure was repeated six times and finally provided about 2 mg. of a waxy solid which stimulated the growth of oat first-internode sections.

## BIOASSAY

The bioassay method of Nitsch and Nitsch (4) was used. Each preparation to be tested was dissolved in 0.2 ml. of acetone in a test tube, and sufficient citrate-phosphate buffer (pH 5.0) containing 2 per cent sucrose was added to bring the volume to 2.0 ml. Ten sections of first internodes of dark-grown oat (*Avena sativa* L. var. Brighton) seedlings, 4 mm. in length, were introduced, and the tubes were rotated for about 20 hours at 1 r.p.m. The oat sections then were removed, and their length was measured with the aid of a photographic enlarger. The resulting data were subjected to statistical analysis by the system of allowances (7).

<sup>3</sup> The technical assistance of Mr. R. V. Berthold and Mr. W. Meudt is gratefully acknowledged.

<sup>4</sup> Ivan Sorvall, Inc., Norwalk, Connecticut.

In general, compounds were tested at several concentrations ranging from 100 p.p.m. to 0.1 p.p.m., corresponding roughly to  $10^{-3}$  to  $10^{-6}$  molar. Similar concentrations of indole-3-acetic acid were used for standardization. Most compounds were tested at each concentration on at least three occasions.

#### SOURCES OF COMPOUNDS

During this investigation, the growth-promoting properties of a wide variety of natural and synthetic long-chain compounds were examined. Most of these substances were obtained from the sources listed in the footnote to Table I, to whom the authors express their appreciation.

2-Methylnonadecan-2-ol, a long-chain tertiary alcohol, was prepared by reaction of recrystallized 2-nonadecanone with methylmagnesium iodide in anhydrous ether, followed by acid hydrolysis, solvent extraction, and crystallization. After several recrystallizations, the compound melted at  $43.5\text{--}44^\circ$  (corr.).

1-Heneicosanol, the normal primary alcohol containing 21 carbon atoms, was prepared by the reaction of purified docosanoic acid with silver nitrate in alcohol to give the silver salt, and further reaction of this compound with bromine in dry carbon tetrachloride to give 1-bromoheneicosane. The bromoalkane was then treated with magnesium and a small amount of methyl iodide in dry ether to give heneicosylmagnesium bromide, which was caused to react with dry oxygen at low temperature. The resulting complex was decomposed with dilute acid, and the product was isolated by filtration. After several recrystallizations, 1-heneicosanol melted at  $64^\circ$  to  $65^\circ$  (corr.).

#### EXPERIMENTAL RESULTS

The growth-promoting material isolated from Maryland Mammoth tobacco was obtained in the form of white, waxy crystals which were soluble in ether, ethanol, and benzene, but apparently insoluble in water. The solid melted at  $70^\circ$  to  $72^\circ$  on a Vanderkamp block, and resolidified only very slowly. Duplicate potassium fusion tests showed that it did not contain nitrogen, sulfur, halogen, or phosphorus. No ultraviolet absorption was observed in concentrated hexane solution with a Cary Model 11 spectrophotometer, indicating the absence of unsaturation.

Infrared spectra were measured on a Perkin-Elmer Model 12A infrared spectrophotometer equipped with an optical condensing system which permitted the use of submilligram samples.<sup>5</sup> As shown in Figure 1, the spectrum of the tobacco isolate is almost identical with that typical of a long-chain normal primary alcohol such as 1-docosanol. The absorption bands at 5.6 and 8.5 microns indicate the presence of a small amount of some carbonyl impurity, probably an ester. Chain branching is not apparent.

<sup>5</sup> The infrared spectral measurements were made by Mr. W. S. Molnar.



TABLE I  
GROWTH PROMOTION BY LONG-CHAIN ALIPHATIC COMPOUNDS  
IN THE OAT FIRST-INTERNODE BIOASSAY

| A. Saturated normal primary alcohols   |          |          |           |            |
|----------------------------------------|----------|----------|-----------|------------|
| Compound                               | Source*  | Activity |           |            |
|                                        |          | 1 p.p.m. | 10 p.p.m. | 100 p.p.m. |
| 1-Dodecanol                            | 1        | —        | —         | —          |
| 1-Tetradecanol                         | 1        | —        | —         | —          |
| 1-Hexadecanol                          | 1        | —        | —         | —          |
| 1-Octadecanol                          | 1,4      | —        | —         | +          |
| 1-Nonadecanol                          | 9        | —        | —         | —          |
| 1-Eicosanol                            | 9        | —        | +         | +          |
| 1-Heneicosanol                         | See text | —        | —         | —          |
| 1-Docosanol                            | 1        | +        | +         | +          |
| 1-Tetracosanol                         | 7        | —        | —         | —          |
| 1-Hexacosanol                          | 7        | —        | —         | —          |
| 1-Octacosanol                          | 7        | —        | —         | —          |
| 1-Triacontanol                         | 7        | —        | —         | —          |
| 1-Dotriacontanol                       | 7        | —        | —         | —          |
| B. Saturated branched primary alcohols |          |          |           |            |
| 18-Methylnonadecan-1-ol                | 7        | —        | —         | —          |
| 18-Methyleicosan-1-ol                  | 7        | —        | —         | —          |
| 20-Methyldocosan-1-ol                  | 7        | —        | —         | —          |
| 22-Methyltricosan-1-ol                 | 7        | —        | —         | —          |
| 22-Methyltetracosan-1-ol               | 7        | —        | —         | —          |
| 24-Methylpentacosan-1-ol               | 7        | —        | —         | —          |
| 24-Methylhexacosan-1-ol                | 7        | —        | —         | —          |
| C. Unsaturated primary alcohols        |          |          |           |            |
| 9-Octadecen-1-ol                       | 1        | —        | —         | —          |
| 9,12-Octadecadien-1-ol                 | 1        | —        | —         | —          |
| 9,12,15-Octadecatrien-1-ol             | 1        | —        | —         | —          |
| Phytol                                 | 8        | +        | +         | +          |
| 9-Eicosen-1-ol                         | 5        | +        | +         | +          |
| 11-Docosen-1-ol                        |          |          |           |            |
| 9-Docosen-1-ol                         | 1        | —        | +         | +          |
| D. Secondary alcohols                  |          |          |           |            |
| 2-Tridecanol                           | 2        | —        | +         | +          |
| 3,9-Diethyltridecan-6-ol               | 10       | —        | —         | —          |
| 2-Heptadecanol                         | 2        | +        | +         | —          |

\* Compounds were obtained from the following sources: 1. Archer-Daniels-Midland Company, Chemical Products Division, Minneapolis, Minnesota. 2. Armour and Company, Research Division, Chicago, Illinois. 3. E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware. 4. Eastman Organic Chemicals Department, Distillation Products Industries, Rochester, New York. 5. Dr. L. A. Goldblatt, Southern Utilization Research and Development Division, U. S. Department of Agriculture, New Orleans, Louisiana. 6. Dr. W. M. Lanham, Research Department, Union Carbide Chemicals Company, South Charleston, West Virginia. 7. Dr. K. E. Murray, Division of Industrial Chemistry, C.S.I.R.O., Melbourne, Australia. 8. Nutritional Biochemicals Corporation, Cleveland, Ohio. 9. Dr. A. W. Scholl, Chemistry Department, Marshall College, Huntington, West Virginia. 10. Union Carbide Chemicals Company, South Charleston, West Virginia.

TABLE I (Continued)

## D. Secondary alcohols (continued)

| Compound               | Source* | Activity |           |            |
|------------------------|---------|----------|-----------|------------|
|                        |         | 1 p.p.m. | 10 p.p.m. | 100 p.p.m. |
| 9-Octadecene-1,12-diol | 1       | —        | —         | —          |
| 1,12-Octadecandiol     | 1       | —        | —         | —          |
| 2-Nonadecanol          | 2       | —        | —         | —          |
| 10-Nonadecanol         | 2       | —        | —         | —          |
| Cholesterol            | 8       | —        | —         | —          |
| Dihydrocholesterol     | 8       | —        | —         | —          |
| 18-Pentatriacontanol   | 2       | —        | —         | —          |

## E. Tertiary alcohol

|                        |          |   |   |   |
|------------------------|----------|---|---|---|
| 2-Methylnonadecan-2-ol | See text | — | — | — |
|------------------------|----------|---|---|---|

## F. Hydrocarbon

|          |   |   |   |   |
|----------|---|---|---|---|
| Eicosane | 4 | — | — | — |
|----------|---|---|---|---|

## G. Ketones

|                           |    |   |   |   |
|---------------------------|----|---|---|---|
| 2-Tridecanone             | 2  | — | — | — |
| 2-Heptadecanone           | 2  | + | + | — |
| 3,9-Diethyltridecan-6-one | 10 | — | — | — |
| 2-Nonadecanone            | 4  | — | — | — |
| 10-Nonadecanone           | 2  | — | — | — |
| 18-Pentatriacontanone     | 2  | — | — | — |

## H. Acids and organic esters

|                             |   |   |   |   |
|-----------------------------|---|---|---|---|
| Octadecanoic acid           | 4 | — | — | — |
| Eicosanoic acid             | 8 | — | — | — |
| Docosanoic acid             | 1 | — | — | — |
| Tetracosanoic acid          | 5 | — | — | — |
| 12-Hydroxyoctadecanoic acid | 1 | — | — | + |
| Octadecyl acetate           | 4 | — | — | — |
| Nonadecyl acetate           | 9 | — | — | — |
| Eicosyl acetate             | 9 | — | — | — |
| Ethylene dihexadecanoate    | 4 | + | + | + |
| Ethylene dioctadecanoate    | 4 | + | + | + |
| Glyceryl trioctadecanoate   | 4 | — | — | — |
| Ethyl octadecanoate         | 4 | — | — | — |

## I. Inorganic esters

|                                      |   |   |   |   |
|--------------------------------------|---|---|---|---|
| Mono- and dioctadecyl acid phosphate | 6 | + | + | + |
| Mono- and didocosyl acid phosphate   | 6 | + | + | + |
| Octadecyl acid sulfate               | 3 | + | + | + |

## J. Amine

|                   |   |   |   |   |
|-------------------|---|---|---|---|
| 1-Aminoöctadecane | 4 | — | — | — |
|-------------------|---|---|---|---|

Comparison of the infrared spectra of the unbranched primary alcohols from 1-decanol to 1-dotriacontanol revealed a regular variation in the ratio of the 13.9 micron absorption to that at 6.8 microns. In addition, it is recognized that the melting points of mixtures of these alcohols are depressed very little below that of the component having the shortest chain length, and that the melting range is indicative of the complexity of the mixture. On this basis, the properties of the tobacco isolate indicate a chain length of between 22 and 28 carbon atoms.

Nilsson, Ryhage, and von Sydow (3) have isolated a neutral, crystalline substance melting at 70° to 74° from air-dried pollen of *Pinus montana*. Mass spectrometric investigation, using a special high-temperature instrument, revealed that this was a mixture of 1-tetracosanol, 1-hexacosanol, and 1-octacosanol. Although mass spectrometric data from the tobacco isolate are inconclusive, it is quite possible that the material consists of a similar mixture.

Discovery of the growth-promoting properties of the long-chain alcohol from tobacco led to an investigation of the dependence of physiological response on chain length, chain branching, and types of functional groups. The compounds tested are listed in Table I, together with their sources and an indication of their activity at three presumed concentrations. Because of the uncertainty of the actual concentrations, no quantitative values have been presented in the table. However, Figures 2, 3, 4, and 5 are histograms which show typical results of the bioassay in cases where growth stimulation was observed for at least one concentration. Relative values such as these were found to be reproducible.

For many years, it has been known that primary alcohols containing more than 18 carbon atoms exhibit an extremely low solubility in water (5), and that they tend to form thin layers at the water-air interface. These facts, together with consideration of the physical aspects of the bioassay method, lead to the conclusion that quantitative interpretation of the data presented in the histograms and in Table I probably should be approached with reservation.

#### DISCUSSION

Long-chain alcohols occur widely throughout the plant kingdom (10). They are found in the free state in the waxy coating of many varieties of fruit such as apple, in the waxy cuticle of stems and leaves, as a coating on the petals of many types of flowers, and in pollen. To a considerable extent, they also have been found to occur in the lipid phase within plant cells. Very little is known about the mechanism of formation of such high molecular weight hydrophobic compounds, the formation of cuticle layers, or the importance of long-chain fatty alcohols to the plants' economy.

The more potent of the long-chain alcohols tested in this series are

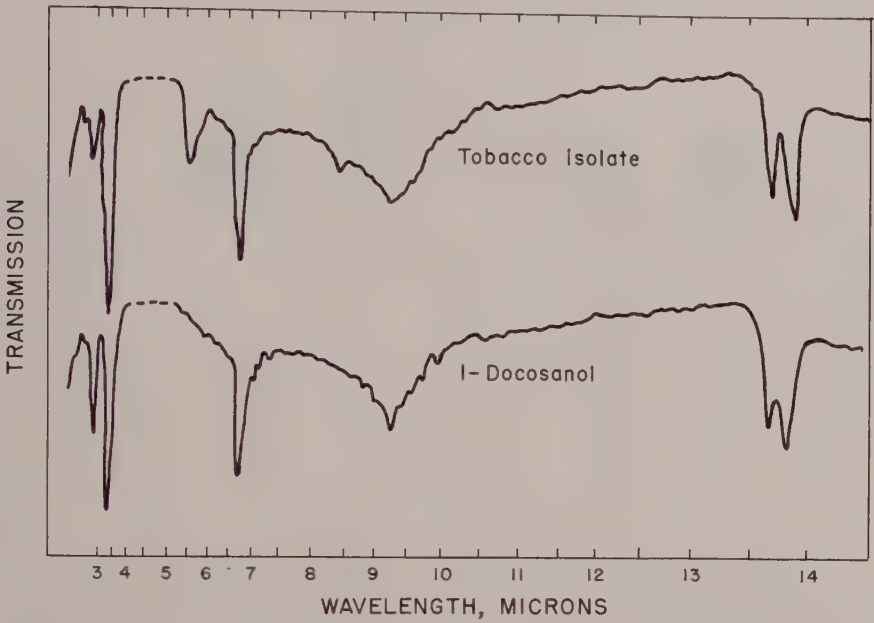


FIGURE 1. Infrared spectra of the substance isolated from Maryland Mammoth tobacco and 1-docosanol.

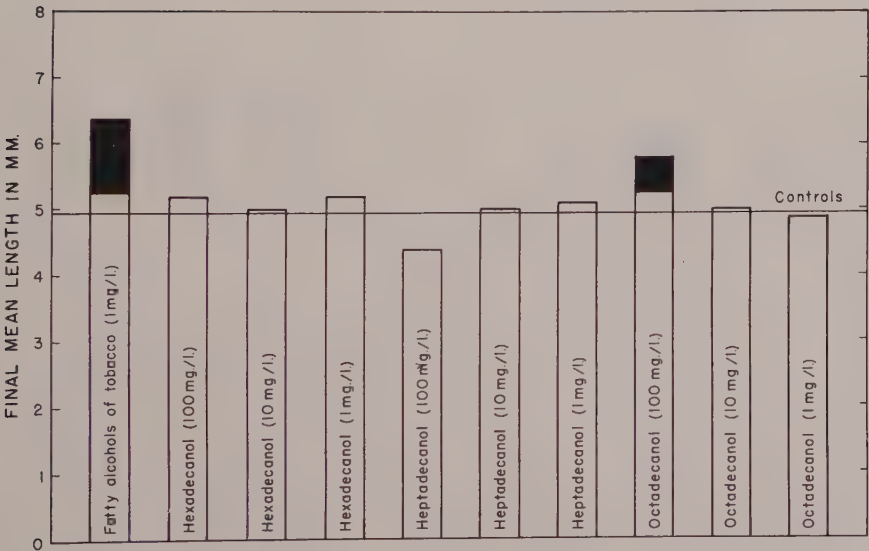


FIGURE 2. Final mean lengths for ten *Avena* first-internode sections floated for 22 hours in buffer (with sucrose) containing the fatty alcohols isolated from tobacco, hexadecanol, heptadecanol, and octadecanol. Shaded areas represent statistically significant differences ( $P=0.01$ ).



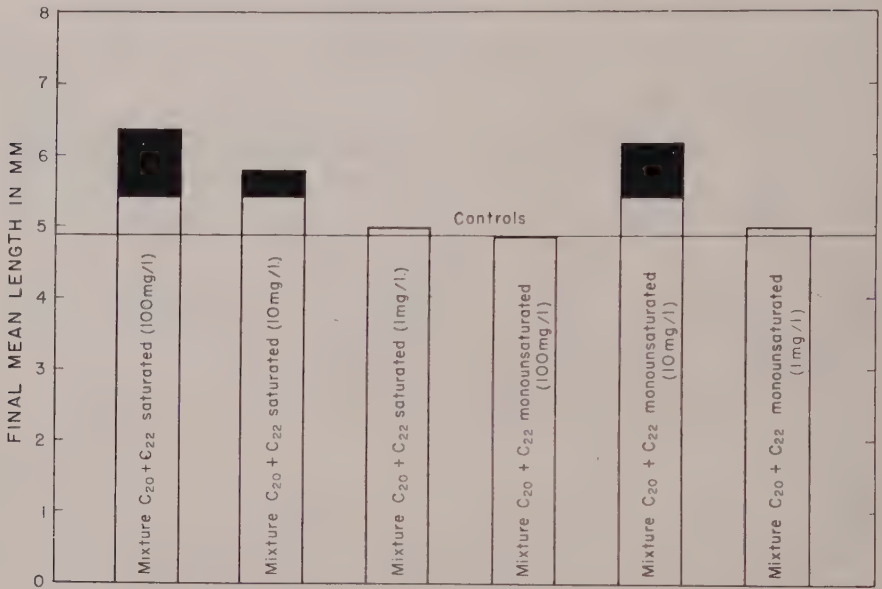


FIGURE 3. Final mean lengths for ten *Avena* first-internode sections floated for 22 hours in buffer (with sucrose) containing C<sub>20</sub>+C<sub>22</sub> saturated alcohols, and C<sub>20</sub>+C<sub>22</sub> unsaturated alcohols. Shaded areas represent statistically significant differences ( $P=0.01$ ).

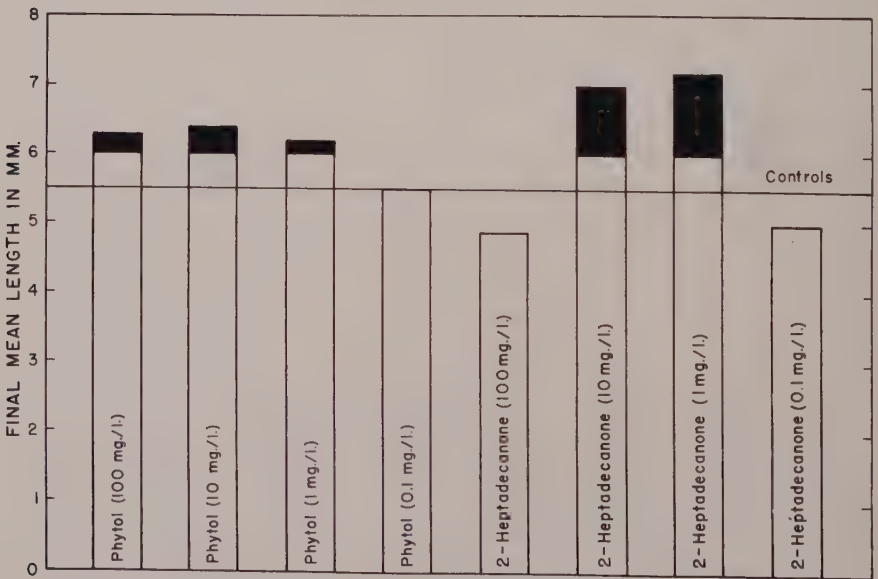


FIGURE 4. Final mean lengths for ten *Avena* first-internode sections floated for 22 hours in buffer (with sucrose) containing phytol and 2-heptadecanone. Shaded areas represent statistically significant differences ( $P=0.01$ ).

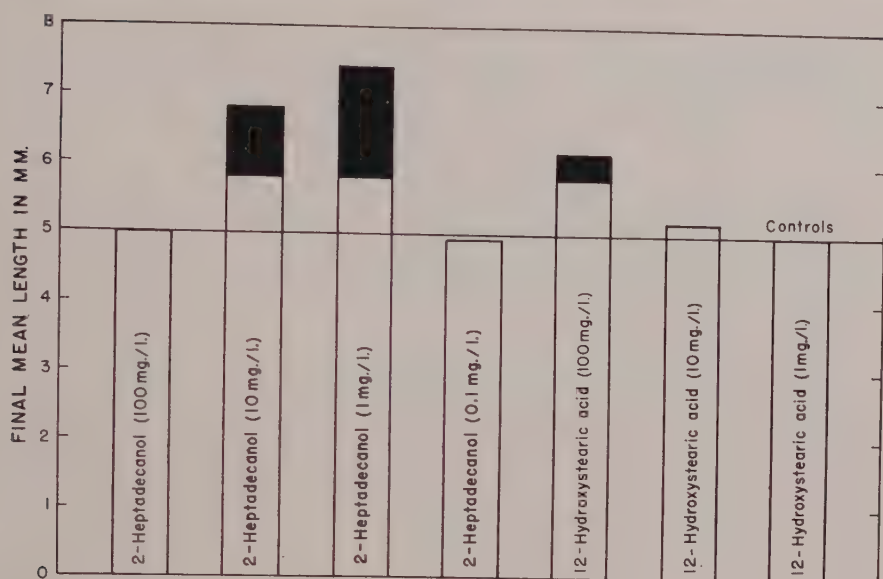


FIGURE 5. Final mean lengths for ten *Avena* first-internode sections floated for 22 hours in buffer (with sucrose) containing 2-heptadecanol and 12-hydroxystearic acid. Shaded areas represent statistically significant differences ( $P=0.01$ ).

about as active as indole-3-acetic acid at comparable concentrations. However, consideration should be given to the insolubility of this type of compound in aqueous media, as mentioned previously. The concept of concentration commonly used in this type of test probably is relatively meaningless here because of inhomogeneity. Such molecules tend to aggregate in thin layers at the water-air interface, and since the oat internode sections used in the bioassay are constantly agitated and brought to the liquid surface, undoubtedly each eventually is covered with a thin, even film of the fatty substance. Variation of concentration may receive quantitative significance through its relation to the thickness of this film. However, the bioassay is at least qualitatively reliable, as shown by the close relation of activity to molecular weight and by the limited types of functional groups which permit growth promotion. The reproducibility of the method is demonstrated by the almost identical results obtained in each of three replicate determinations conducted during successive weeks.

From the data of Table I, it is apparent that a number of alcohols containing from 17 to 22 carbon atoms, and their acidic esters, possess pronounced activity. Hydrocarbons, neutral esters, acids, halides, and other alcohols are essentially inactive. The surprising potency of methyl pentadecyl ketone might be explained by its easy reduction to the corresponding (highly active) alcohol. The mechanism by which such compounds promote plant growth is obscure. The inactivity of the  $C_{19}$  and  $C_{21}$  primary al-

cohols suggests that oxidation to the corresponding carboxylic acid might be the first step. However, whereas the  $C_{18}$ ,  $C_{20}$ , and  $C_{22}$  alcohols are effective growth promoters, the corresponding carboxylic acids are completely inactive. The high degree of activity exhibited by the acid sulfate esters and acid phosphate esters may present a key to the mode of action. Some surface-active agents affect bud formation and development (2), and it is possible that in the present case, too, this property offers a common basis for activity. Stowe (6) has reported the stimulation of growth in pea epicotyl sections by several long-chain compounds, but only in the presence of added indole-3-acetic acid.

In connection with the growth of Maryland Mammoth tobacco, Chibnall and coworkers (1) have reported that the neutral fraction of cellular wax from the Connecticut variety of tobacco contains only hydrocarbons. The absence of hydrocarbons in Maryland Mammoth tobacco, and the occurrence instead of the corresponding long-chain alcohols, may present a significant biochemical difference between the Mammoth and other types. It also leads us to speculate that growth in plants may be governed by a more or less complicated system of regulators rather than by a single auxin such as indole-3-acetic acid. Further work directed toward description of the hormone complex operating in Maryland Mammoth tobacco is now in progress.

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# EVALUATION OF 2-(3,5-DICHLORO-2-BIPHENYLYLOXY)- TRIETHYLAMINE AS AN INSECTICIDE ADJUVANT<sup>1</sup>

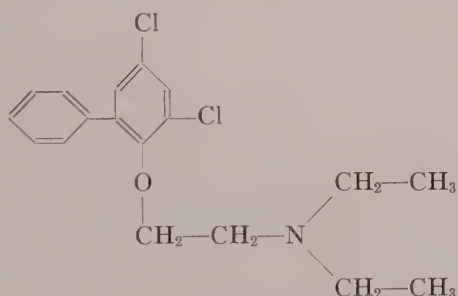
HERBERT H. MOOREFIELD AND EDWIN R. TEFFT

## SUMMARY

2-(3,5-Dichloro-2-biphenylyloxy)triethylamine, which extends the activity of a diverse series of unrelated drugs through inhibition of metabolic transformation, was tested as an insecticide synergist. The compound did not potentiate the insecticidal properties of the pyrethrins, allethrin, cyclothrin, DDT, Diazinon, or malathion; nor did it inhibit DDT-dehydrochlorinase, the detoxication enzyme from DDT-resistant house flies. Synergistic responses were demonstrated with mixtures of the amine and 3-*tert*.-butylphenyl *N*-methylcarbamate, Sevin®, Pyrolan, and Isolan. These results indicate that synergism of the pyrethrins and the carbamates is not a correlative phenomenon and that although the methylenedioxyphenyl compounds have been previously shown to extend the action of both classes of toxicants, specific carbamate synergists are feasible.

## INTRODUCTION

The potentiating agent, 2-(3,5-dichloro-2-biphenylyloxy)triethylamine (Lilly 18947):



has been shown to prolong the pharmacological effects of a number of drugs with widely divergent chemical structures. Its activity has been ascribed to the inhibition of various unrelated enzyme systems which metabolically alter the drug, thus promoting an extension of activity. Several of the biochemical pathways that have been reportedly blocked are oxidation of the side-chain of hexobarbital, dealkylation of aminopyrine, deamination of amphetamine, cleavage of the codeine ether linkage, hydroxylation of acetanilide, and the conjugation of morphine (5).

Practices in the field of modern insect control present certain parallels. Synergistic mixtures of chemicals are widely employed for the purpose of extending insecticidal activity. The synergists of the methylenedioxyphenyl series, although primarily used in combination with the pyrethrins,

<sup>1</sup> This investigation was sponsored by a Union Carbide Chemicals Company Fellowship.



have been reported to exert similar responses with other classes of insecticides, e.g., chlorinated hydrocarbons (6, 14, 17), rotenoids (1), phosphates (3, 8, 15), and carbamates (4, 10). Although the mode of action of these synergists has not been completely elucidated, various researchers have interpreted their results in terms of the prevention of detoxication (2, 7, 19, 20).

The present work describes a series of experiments designed to determine if the multiple drug-potential effects of 2-(3,5-dichloro-2-biphenyloxy)triethylamine could be demonstrated as a synergistic response in admixture with various insecticides.

#### EXPERIMENTAL

*Insecticidal properties.* In order for true synergism to be established, the insecticidal properties of each component of the combination must be accurately assessed. 2-(3,5-Dichloro-2-biphenyloxy)triethylamine displayed no insect toxicity in a series of evaluation tests in which excess amounts of the compound were employed.

Nasturtium (*Tropaeolum majus* L.) plants, infested with the bean aphid (*Aphis fabae* Scop.), were sprayed until run-off with a solution of the amine (1000 p.p.m. formulated in 1:10 acetone-water plus emulsifier) without effective control. Similar application to bean (*Phaseolus vulgaris* L., var. Tendergreen) foliage containing moderate populations of the two-spotted mite (*Tetranychus telarius* L.) caused no mortality. In feeding tests, both the Mexican bean beetle (*Epilachna varivestris* Muls.) and the Southern armyworm (*Prodenia eridania* Cram.) consumed bean leaves dipped in the above solution without any resultant kill. The compound was also essentially innocuous to the house fly (*Musca domestica* L.) as individuals treated topically with doses of 20  $\mu$ g. per  $\mu$ l. of acetone were unaffected.

*Combination with pyrethrins insecticides.* Topical applications of microdrops containing pyrethrins, allethrin (3-allyl-2-methyl-4-oxo-2-cyclopenten-1-yl chrysanthemumate), or cyclothrin (3-[2-cyclopenten-1-yl]-2-methyl-4-oxo-2-cyclopenten-1-yl chrysanthemumate) in acetone, onto the pronotum of 3-day-old, adult, female house flies were made with a micrometer-driven syringe. Comparative tests in which these compounds were also mixed in various proportions (1:10 to 10:1) with 2-(3,5-dichloro-2-biphenyloxy)triethylamine indicated that no synergism was effected. These negative findings were verified in large Peet-Grady evaluations (13).

*Combination with DDT.* No enhanced activity of DDT (1,1,1-trichloro-2,2-bis[*p*-chlorophenyl]ethane), fortified with 2-(3,5-dichloro-2-biphenyloxy)triethylamine, could be demonstrated with DDT-susceptible house flies (NAIDM strain, DDT LD<sub>50</sub> = 0.25  $\mu$ g./fly) in topical treatments. Similar tests with DDT-resistant flies (DDT-45 strain, developed at U.S. Public Health Laboratories, Savannah, Georgia) did not indicate that the

amine would in any measure restore the effectiveness of the insecticide to tolerant species.

The detoxication enzyme, DDT-dehydrochlorinase, prepared as breis of DDT-resistant flies, was not inhibited by the amine hydrobromide salt in *in vitro* assays (16).

*Combination with phosphate insecticides.* Mixed doses of the amine and malathion (*S*-[1,2-bis(ethoxycarbonyl)ethyl]*O,O*-dimethyl phosphorodithioate) or Diazinon (*O,O*-diethyl *O*-[2-isopropyl-4-methyl-6-pyrimidinyl] phosphorothiate) applied topically to house flies were no more effective

TABLE I

SYNERGISM OF THE CARBAMATE INSECTICIDES WITH 2-(3,5-DICHLORO-2-BIPHENYLOXY)-TRIETHYLAMINE IN TOPICAL TREATMENT TO THE HOUSE FLY

| Compound                                                   | Dose, $\mu$ g.<br>(in 1 $\mu$ l.<br>acetone/fly) | % Mortality                  |                                              |
|------------------------------------------------------------|--------------------------------------------------|------------------------------|----------------------------------------------|
|                                                            |                                                  | Compound<br>applied<br>alone | Compound<br>applied with<br>5 $\mu$ g. amine |
| 2-(3,5-Dichloro-2-biphenyloxy)triethylamine                | 5.0                                              | 0                            | —                                            |
| 3- <i>tert</i> .-Butylphenyl <i>N</i> -methylcarbamate     | 0.3                                              | 5                            | 100                                          |
| 1-Naphthyl <i>N</i> -methylcarbamate (Sevin)               | 1.0                                              | 8                            | 95                                           |
| 1-Phenyl-3-methylpyrazolyl-5-dimethylcarbamate (Pyrolan)   | 0.1                                              | 0                            | 100                                          |
| 1-Isopropyl-3-methylpyrazolyl-5-dimethylcarbamate (Isolan) | 0.3                                              | 4                            | 100                                          |

than equivalent doses of the phosphate insecticide presented alone. Ratios of adjuvant to toxicant were varied from equal proportions to 10:1, all of which failed to elicit synergism.

*Combination with carbamate insecticides.* Significant synergism was produced in evaluations of 2-(3,5-dichloro-2-biphenyloxy)triethylamine mixed with the carbamate insecticides. Applied topically to the house fly in 1  $\mu$ l. volumes of acetone, potentiation responses were evident with each of a series of structurally different carbamates. At concentrations in which each of the combined compounds were individually ineffective as fly toxicants, mixed doses of the amine with a phenyl, a bicyclic, and two heterocyclic carbamates produced approximately 100 per cent mortality. Sevin® (1-naphthyl *N*-methylcarbamate), Pyrolan (1-phenyl-3-methylpyrazolyl-5-dimethylcarbamate), Isolan (1-isopropyl-3-methylpyrazolyl-5-dimethylcarbamate), and 3-*tert*.-butylphenyl *N*-methylcarbamate were all efficiently potentiated. These results are shown in Table I.

*Cholinesterase assays.* The anti-esterase properties of 2-(3,5-dichloro-2-biphenyloxy)triethylamine hydrobromide were estimated with house fly head cholinesterase, red blood cell cholinesterase, and the pseudoesterase from blood serum. Methods of enzyme preparation, standardization, and

TABLE II  
COMPARATIVE AFFINITY OF 2-(3,5-DICHLORO-2-BIPHENYLOXY)-  
TRIETHYLAMINE HYDROBROMIDE FOR VARIOUS ESTERASES

| Enzyme source        | I <sub>50</sub> (molar concentration required to effect 50% enzyme inhibition) |
|----------------------|--------------------------------------------------------------------------------|
| House fly head       | $1.5 \times 10^{-6}$                                                           |
| Human red blood cell | $1.7 \times 10^{-4}$                                                           |
| Human blood serum    | $6.4 \times 10^{-5}$                                                           |

manometric assay have been previously described (12). The amine moderately inhibited the esterases from all three sources. Comparative results of this study are presented in Table II.

#### DISCUSSION

Although the nature of the inhibitory effects described for 2-(3,5-dichloro-2-biphenyloxy)triethylamine in combination with various drugs is quite polyvalent, the synergistic response in mixture with insecticides appears to be rather specific. If the activity of the pyrethrins adjuvants is one of antagonizing detoxication (2, 7, 19, 20), the amine failed to interfere with the mechanism.

The mode of action of DDT is not understood, but house flies resistant to this compound have been shown to contain high levels of a detoxifying enzyme, DDT-dehydrochlorinase (16). The joint action of some of the structural analogues of DDT, when utilized in combination with the toxicant, results in a restoration of the efficacy of the insecticide (9, 18). These compounds function *in vitro* as competitive inhibitors of DDT-dehydrochlorinase (11), and although certain topological similarities between the so-called DDT-synergists and the substituted triethylamine are evident, the latter chemical is completely inactive in this capacity. Likewise, no potentiation of the DDT activity could be detected with topical, mixed doses on either susceptible or resistant flies.

Prior studies of carbamate synergism have pointed out the effectiveness of the pyrethrins extenders when used with the carbamic acid esters (10). The fact that 2-(3,5-dichloro-2-biphenyloxy)triethylamine is impotent in combinations with the pyrethrins, yet potentiates the carbamates, demonstrates that a strict correlation between upgrading the activity of these insecticides need not be anticipated. Although the methylenedioxyphenyl compounds such as piperonyl butoxide ( $\alpha$ -[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene), sesamex (2-[2-ethoxyethoxy]ethyl 3,4-methylenedioxyphenyl acetal of acetaldehyde), etc. are active with both the pyrethrins and carbamates, molecules of vastly different structure and properties are likely to be found that will individually augment the performance of each of these classes of toxicants. Selec-

tive testing may well produce a specific, economically-attractive, carbamate synergist.

The property of the amine to inhibit cholinesterase has not previously been reported from pharmacological studies. However, this feature has no apparent role in its performance as a carbamate synergist because such responses were not noted when tested in combination with another class of efficient anticholinesterase insecticides, the organic phosphates.

#### ACKNOWLEDGMENT

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# FUNGICIDAL EFFECTIVENESS OF S,S'-DIMETHYL XANTHIOGENETHYLENEBIS[DITHIOCARBAMATE] AND RELATED COMPOUNDS

DEWAYNE C. TORGESON,<sup>1</sup> J. A. LAMBRECH,<sup>2</sup> AND W. H. HENSLEY<sup>2</sup>

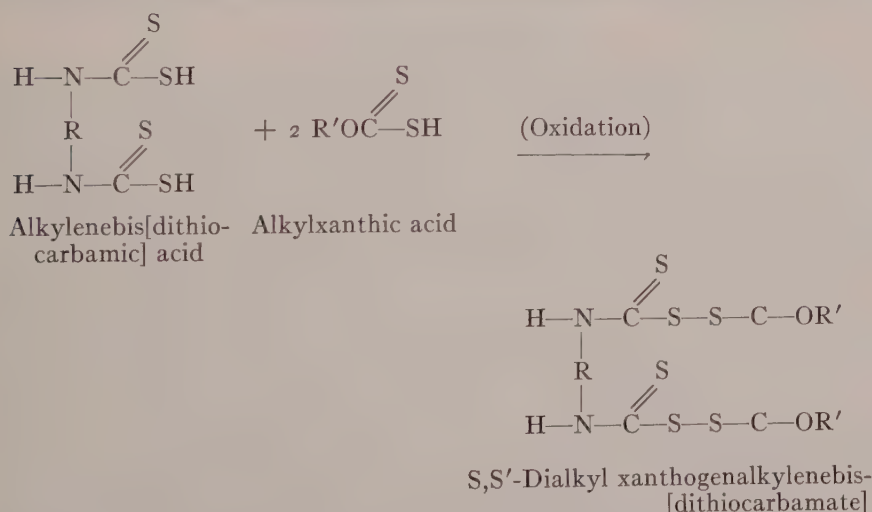
## SUMMARY

Using inhibition of spore germination and control of early blight and late blight of tomato as assay methods, the fungicidal effectiveness of a series of S,S'-dialkyl xanthogenalkylenebis[dithiocarbamates] was studied. The methyl, ethyl, isopropyl, and butyl homologues were about equally fungitoxic but toxicity was reduced when the alkyl group was increased to cyclohexyl or 2-ethylhexyl. Increasing the alkylene group above ethylene also resulted in a loss of activity. A disulfide linkage was more active than the tri- or tetrasulfide.

On the basis of preliminary field test results, S,S'-dimethyl xanthogenethylenbis[dithiocarbamate] was selected for more extensive field tests and found to be as effective as zineb (zinc ethylenebis[dithiocarbamate]) for the control of potato late blight.

## INTRODUCTION

Derivatives of dithiocarbamic acid, in particular the zinc and manganese ethylenebis[dithiocarbamates] and zinc and ferric dimethyldithiocarbamates, are widely used as foliage fungicides. The alkylxanthates, on the other hand, although highly fungitoxic (3, 5) are too phytotoxic for use as foliage fungicides. A new group of safe and fungicidally effective compounds, the S,S'-dialkyl xanthogenalkylenebis[dithiocarbamates], in which the xanthate and dithiocarbamate groups are combined is discussed in this report. They were obtained by the following general type reaction (6):



R indicates an alkylene group  
R' indicates an alkyl or cyclic group

<sup>1</sup> Plant Pathologist, Boyce Thompson Institute for Plant Research, Inc., Union Carbide Chemicals Company Fellowship.

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## METHODS

## LABORATORY AND GREENHOUSE EVALUATION METHODS

The fungitoxicity of the compounds discussed in this paper was determined using spore inhibition and foliage protection evaluation techniques.

Inhibition of spore germination was measured by the test tube dilution method (1) using spores of *Alternaria oleracea* Milb. and *Monilinia fructicola* (Wint.) Honey. Test chemicals were formulated as solutions or suspensions at an initial concentration of 1000 p.p.m. by adding a solvent such as acetone and an emulsifier (Tergitol NPX, an alkyl phenyl polyethylene glycol ether) in sufficient volume to give a final concentration of 5 and 0.01 per cent, respectively. Using a tenfold dilution series, the ED<sub>50</sub> ranges were determined and given an alphabetical rating (11).

The protective fungicidal ability of the test chemicals was assessed using the early blight, *Alternaria solani* (Ell. & Mart.) Jones & Grout, and late blight, *Phytophthora infestans* (Mont.) DeBary, fungus diseases of tomato (*Lycopersicon esculentum* Mill.) (7, 8). Chemicals were formulated for testing as above except that the starting concentration was 2000 p.p.m. They were evaluated in a fivefold dilution series. The ED<sub>95</sub> value of each material was determined by interpolation from the curve obtained when the percentage disease control data were plotted on logarithmic-probability paper.

## FIELD EVALUATION METHODS

The more fungicidally active compounds were evaluated as foliage fungicides under field conditions during the springs of 1954 and 1955. These trials were conducted by G. R. Townsend on muck soil at Florida Field Trials, Belle Glade, Florida (9). Evaluations of the test compound's effectiveness in protecting potatoes (*Solanum tuberosum* L.) against blight caused by *Phytophthora infestans* and celery (*Apium graveolens* L.) against early blight caused by *Cercospora apii* Fres. were made. Spray intervals somewhat longer than those normally used for commercial disease control were used in order to more readily detect differences between test chemicals.

For the test conducted in the spring of 1954, potatoes of the variety Bliss Triumph were planted in rows spaced four feet apart. Plots consisted of a 50-foot strip of row and each treatment was replicated four times. Four compounds, formulated as 70 per cent active ingredient wettable powders, were tested. Rates of 0.5, 1, and 2 pounds of active ingredient per hundred gallons of spray mixture were compared with zineb, freshly prepared from nabam (ethylenebis[dithiocarbamate]) and zinc sulfate. An unsprayed control treatment was also included. Applications were made with

a single row sprayer equipped with four nozzles at 300 pounds of pressure on March 10, 15, 22, 30, April 6, 12, and 19. Rates of 140 to 150 gallons per acre were applied in the first four applications and 160 to 175 gallons per acre in later applications. Visual estimates of the percentage of foliage damaged by late blight in each plot were made on April 23. The tubers were dug and yields determined on May 8.

In the spring of 1955, a test, identical with the one described above but with the differences noted below, was conducted. Potatoes of the Pontiac variety planted on February 18 served as the test plants. One compound was compared with zineb, at rates of 0.5 and 1 pound per 100 gallons of spray mixture. Applications were made on March 10, 17, 23, 30, April 5, 11, 15, 19, and 25 at 250 to 300 pounds pressure at a rate of 150 gallons per acre. Blight estimates were made April 26, and the potatoes were harvested and yields measured on May 12.

Celery of the Supreme Golden variety was used in the 1954-1955 celery late blight control trials. The plants were set in the field on December 3, 1954 in rows with a 4-foot spacing. Treatments were the same as in the 1955 potato late blight trial described above. The test materials were applied on December 16, 23, 30, January 6, 12, 20, 26, February 2, 9, 16, 23, March 2, and 9 at a rate of 100 gallons per acre in the first eight applications and of 150 gallons per acre in the last four applications at 200 to 300 pounds pressure. Blight damage estimates were made March 16 and on March 18 the celery was harvested and yield data taken.

## EXPERIMENTAL RESULTS

### LABORATORY AND GREENHOUSE STUDIES

The S,S'-dialkyl xanthogenalkylenebis[dithiocarbamates] differed only slightly in their fungitoxicity to spores of *Alternaria oleracea* and *Monilinia fructicola* in the slide-germination test (Tables I and II). The ED<sub>50</sub> ranges were relatively high and not well correlated with their performance as foliage protectants for the control of *Alternaria solani* and *Phytophthora infestans* on tomato.

Changes in the alkyl radical of the xanthate portion of those derivatives prepared from ethylenebis[dithiocarbamate] had a pronounced effect on their foliage protectant activities (Table I). The methyl, ethyl, isopropyl, and butyl derivatives were similar in their fungicidal effectiveness. Activity was reduced when the alkyl chain was lengthened to 2-ethylhexyl and the cyclohexyl derivative was even less active. Differences in fungicidal activity were even more striking when the alkylene group of S,S'-dimethyl xanthogenalkylenebis[dithiocarbamate] was modified (Table I). The ethylene derivative was much more active than the propylene-1,2, and hexamethylene homologues. When the di-, tri, and tetrasulfides were com-



TABLE I  
FUNGICIDAL EFFECTIVENESS OF S,S'-DIALKYL XANTHOGEN-  
ALKYLENEBIS[DITHIOCARBAMATES]

| $\begin{array}{c} \text{S} \\ \parallel \\ \text{HNC}-\text{SS}-\text{COR}' \\   \\ \text{R} \\   \\ \text{HNC}-\text{SS}-\text{COR}' \\   \\ \text{R} \end{array}$ |                                                                                                                                                                | Spore inhibition,<br>ED <sub>50</sub> range* |                               | Tomato blight control, ED <sub>95</sub> in p.p.m. |                               |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|-------------------------------|---------------------------------------------------|-------------------------------|
| R                                                                                                                                                                   | R'                                                                                                                                                             | <i>A.</i><br><i>oleracea</i>                 | <i>M.</i><br><i>fruticola</i> | <i>A.</i><br><i>solani</i>                        | <i>P.</i><br><i>infestans</i> |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | —CH <sub>3</sub>                                                                                                                                               | C                                            | B                             | 10                                                | 15                            |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | —CH <sub>2</sub> CH <sub>3</sub>                                                                                                                               | B                                            | B                             | 20                                                | 15                            |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | —CH(CH <sub>3</sub> ) <sub>2</sub>                                                                                                                             | B                                            | B                             | 20                                                | 10                            |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | —CH <sub>2</sub> (CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub>                                                                                               | C                                            | B                             | 20                                                | 10                            |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | $\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{—CH}_2\text{CH}(\text{CH}_2)_3\text{CH}_3 \end{array}$                                                    | B                                            | B                             | 80                                                | 40                            |
| —CH <sub>2</sub> CH <sub>2</sub> —                                                                                                                                  | $\begin{array}{c} \text{CH}_2-\text{CH}_2 \\ / \quad \backslash \\ \text{—CH} \qquad \text{CH}_2 \\ \backslash \quad / \\ \text{CH}_2-\text{CH}_2 \end{array}$ | C                                            | B                             | 400                                               | 70                            |
| —CH <sub>2</sub> (CH <sub>2</sub> ) <sub>4</sub> CH <sub>2</sub> —                                                                                                  | —CH <sub>3</sub>                                                                                                                                               | B                                            | B                             | > 2000                                            | 400                           |
| $\begin{array}{c} \text{—CH}_2-\text{CH—} \\   \\ \text{CH}_3 \end{array}$                                                                                          | —CH <sub>3</sub>                                                                                                                                               | C                                            | B                             | 200                                               | 80                            |

\* B = 10–100 p.p.m., C = 100–1000 p.p.m.

pared, the disulfide was found to be the most effective derivative (Table II).

Circumstantial evidence indicated that these compounds were not cleaving at the disulfide linkage to yield the parent xanthate and dithiocarbamate, both of which would be fungicidally active. Such a cleavage would, for example, in the case of S,S'-dimethyl xanthogenethylenebis[dithiocarbamate] free methylxanthate and ethylenebis[dithiocarbamate], both of which are more phytotoxic than the parent compound.

#### FIELD STUDIES

Field test data were analyzed statistically using the standard analysis of variance, Tukey's method of multiple comparisons (10) or Wilcoxon's rank methods<sup>3</sup> (12). In each case, the method most applicable for that particular set of data was employed.

<sup>3</sup> Wilcoxon, Frank. Unpublished methods.

TABLE II  
FUNGICIDAL EFFECTIVENESS OF THE SULFIDES OF DIMETHYL  
XANTHOGENETHYLENEBIS[DITHIOCARBAMATE]

| $\begin{array}{c} \text{S} \\ \parallel \\ \text{HNC}-\text{S}_n-\text{COCH}_3 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{S} \\ \parallel \\ \text{HNC}-\text{S}_n-\text{COCH}_3 \end{array}$ | Spore inhibition,<br>ED <sub>50</sub> range* |                      | Tomato blight control,<br>ED <sub>95</sub> in p.p.m. |                     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|----------------------|------------------------------------------------------|---------------------|
|                                                                                                                                                                                                          | <i>A. oleracea</i>                           | <i>M. fructicola</i> | <i>A. solani</i>                                     | <i>P. infestans</i> |
| n = 2                                                                                                                                                                                                    | C                                            | B                    | 10                                                   | 15                  |
| n = 3                                                                                                                                                                                                    | B                                            | B                    | 400                                                  | 200                 |
| n = 4                                                                                                                                                                                                    | C                                            | B                    | 300                                                  | 300                 |

\* B = 10–100 p.p.m., C = 100–1000 p.p.m.

Comparisons of the methyl, ethyl, isopropyl, and butyl derivatives of S,S'-dialkyl xanthogenethylenebis[dithiocarbamate] with zineb for the control of potato late blight in 1954 showed that with the exception of the butyl derivatives they were equally effective (Table III). Neither the ethyl nor butyl homologue increased the yields as much as zineb.

TABLE III

EFFECTIVENESS OF FOUR S,S'-DIALKYL XANTHOGENETHYLENEBIS[DITHIOCARBAMATES]  
FOR THE CONTROL OF POTATO LATE BLIGHT, BELLE GLADE, FLORIDA, 1954

| Compound                                                    | Rate,<br>lbs./100 gal. | Average %<br>disease | Total<br>yield,<br>lbs. |
|-------------------------------------------------------------|------------------------|----------------------|-------------------------|
| S,S'-Dimethyl xanthogenethylene-<br>bis[dithiocarbamate]    | 0.5                    | 33.7                 | 355                     |
|                                                             | 1                      | 23.7**               | 344                     |
|                                                             | 2                      | 11.2**               | 363*                    |
| S,S'-Diethyl xanthogenethylene-<br>bis[dithiocarbamate]     | 0.5                    | 32.5*                | 312†                    |
|                                                             | 1                      | 20.0**               | 336                     |
|                                                             | 2                      | 15.0**               | 339†                    |
| S,S'-Diisopropyl xanthogenethylene-<br>bis[dithiocarbamate] | 0.5                    | 36.2                 | 346*                    |
|                                                             | 1                      | 25.0*                | 367*                    |
|                                                             | 2                      | 15.0*                | 377*                    |
| S,S'-Dibutyl xanthogenethylene-<br>bis[dithiocarbamate]     | 0.5                    | 37.5                 | 335                     |
|                                                             | 1                      | 36.2                 | 310††                   |
|                                                             | 2                      | 31.2**††             | 314††                   |
| Zineb                                                       | 0.5                    | 37.5                 | 363**                   |
|                                                             | 1                      | 13.7                 | 405**                   |
|                                                             | 2                      | 5.0**                | 422**                   |
| Control                                                     | —                      | 53.7                 | 290                     |

\* Different from the control at the 5 per cent level.

\*\* Different from the control at the 1 per cent level.

† Different from zineb at the 5 per cent level.

†† Different from zineb at the 1 per cent level.

TABLE IV

EFFECTIVENESS OF S,S'-DIMETHYL XANTHOGENETHYLENEBIS[DITHIOCARBAMATE]  
FOR THE CONTROL OF POTATO LATE BLIGHT AND CELERY EARLY BLIGHT,  
BELLE GLADE, FLORIDA, 1955

| Compound                                                 | Rate,<br>lbs./100<br>gal. | Potato late blight      |                         | Celery early blight     |                         |
|----------------------------------------------------------|---------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
|                                                          |                           | Average<br>%<br>disease | Total<br>yield,<br>lbs. | Average<br>%<br>disease | Total<br>yield,<br>lbs. |
| S,S'-Dimethyl xanthogenethylene-<br>bis[dithiocarbamate] | 0.5                       | 16.2                    | 495                     | 5.0                     | 607                     |
|                                                          | 1                         | 13.7                    | 458                     | 1.7                     | 652                     |
| Zineb                                                    | 0.5                       | 20.0                    | 450                     | 0.7                     | 671                     |
|                                                          | 1                         | 8.7                     | 483                     | 0.5                     | 693                     |
| Control                                                  | —                         | 38.7                    | 414                     | 33.7                    | 479                     |

On the basis of its general performance and potential manufacturing economics S,S'-dimethyl xanthogenethylenebis[dithiocarbamate] was selected for additional field evaluation in 1955. Using a formulation which appeared in the laboratory to be superior to that evaluated the previous year the methyl derivative was fully as effective as zineb for the control of potato late blight and at the low dosage rate was the only treatment giving yields significantly better than the control (Table IV). Somewhat different results were obtained on celery (Table IV). Although the blight control effected by the experimental xanthate as compared with the control was statistically significant its performance did not equal that of zineb. Zineb treated plots were also more productive.

#### DISCUSSION

Combining ethylenebis[dithiocarbamate] with an alkylxanthate to yield S,S'-dialkyl xanthogenethylenebis[dithiocarbamate] resulted in a new group of highly effective foliage fungicides. The methyl, ethyl, isopropyl, and butyl xanthate homologues were the most toxic with activity being reduced as the alkyl group was increased in size in the cyclohexyl and 2-ethylhexyl derivatives. These findings are in agreement with those of Davies and Sexton (3) who found that fungitoxicity was decreased as the alkyl group of a homologous series of alkylxanthates was lengthened. Likewise, increasing the alkylene group of the alkylenebis[dithiocarbamate] portion of the molecule above ethylene reduced activity in accord with the work of Barratt and Horsfall (2). The disulfide linkage was more effective than the tri- or tetrasulfide linkage which is also in agreement with their findings with ethylenethiuram disulfide and tetrasulfide.

Activity of the methyl, ethyl, isopropyl, and butyl derivatives was quite similar in the field for the control of potato late blight with the methyl and isopropyl derivatives being somewhat more active. S,S'-

Dimethyl xanthogenethylenebis[dithiocarbamate] which was selected for additional field tests performed well in the control of potato late blight but was less efficient than zineb for the control of celery early blight in Florida. However, when evaluated against potato early blight in West Virginia under code (7443) results were somewhat less favorable (4).

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# ALTERED BIOCHEMICAL PATTERNS INDUCED IN TOBACCO BY CUCUMBER MOSAIC VIRUS INFECTION, BY THIOURACIL, AND BY THEIR INTERACTION

CLARK A. PORTER AND LEONARD H. WEINSTEIN

## SUMMARY

Vamorr 48 tobacco (*Nicotiana tabacum* L.) plants grown in nutrient solutions were subjected to infection with cucumber mosaic virus, to treatment with 5 p.p.m. of thiouracil, and to a combination of both. Leaves were analyzed for proteins, free amino acids and amides, nucleic acids, and malic and citric acids at 0, 72, 230, and 286 hours after initiation of the experiment. In general, the amount of the various constituents in the control plants increased during the experiment. However, ribonucleic acid decreased during the interval that protein was accumulating at a maximum rate. There was an increase in deoxyribonucleic acid during this time.

Virus inoculations were made 48 hours after the beginning of the experiment. The subsequent infection caused a considerable increase in glutamine within 24 hours but had little effect on the level of other components for 182 hours. Between this time and 238 hours, infection produced considerable reductions in all constituents except ribonucleic and deoxyribonucleic acids. The amounts of these remained nearly equivalent in the control and infected leaves.

Thiouracil treatment singly or in combination with virus infection resulted in decreases in all constituents except the free amino- and amide-nitrogen pool. The increase in this case was relatively large and due primarily to an inhibition of protein synthesis rather than proteolysis.

The present data substantiate earlier conclusions that thiouracil interferes with protein and nucleic acid metabolism in tobacco and that it does not materially impair the biosynthesis of amino acids.

## INTRODUCTION

It was shown previously that major biochemical changes were induced in tobacco plants by infection with cucumber mosaic virus (CMV) and in both noninfected and infected tobacco plants by treatment with thiouracil (12). CMV infection alone apparently stimulated host metabolism since it caused an increase in the amounts of certain constituents. Thiouracil treatment of both noninfected and infected plants decreased the levels of protein nitrogen and nucleic acid phosphorus while it increased the amounts of total alcohol-soluble amino- and amide-nitrogen. This indicated that thiouracil had interfered in some manner with the incorporation of amino acids into proteins but had not materially impaired the capacity of the plants to produce amino acids and amides. It was suggested that a block occurred in protein synthesis because of an aberration in nucleic acid metabolism resulting from the antagonism of uracil by thi-

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ouracil. The earlier investigation did not yield information concerning biochemical changes occurring in the tobacco plants between the initiation and conclusion of the experiment. This paper reports the results of additional studies designed to elucidate certain biochemical changes at intervals during a 286-hour period in tobacco plants subjected to CMV infection, thiouracil treatment, and a combination of the two.

#### MATERIALS AND METHODS

Vamorr 48 tobacco (*Nicotiana tabacum* L.) plants were grown by the methods described previously (12). The experiment was initiated by transferring the plants to nutrient cultures containing 0 or 5 p.p.m. of thiouracil. One-half of the total number of plants in each concentration of thiouracil were inoculated with CMV 48 hours later. All of the inoculated plants had characteristic CMV symptoms after 3 or 4 days. Samples comprised of all of the leaves from two plants were collected from each thiouracil treatment at 0, 72, 230, and 286 hours after initiation of the experiment. Each leaf sample was weighed and immediately homogenized in 80 per cent ethanol with a VirTis "45" Homogenizer. The ethanol was decanted from the residue following centrifugation, and the residue was extracted four additional times with fresh aliquots of 80 per cent ethanol. All five extracts from each sample were combined, and the extracted residues were lyophilized.

The ethanol extract from each sample was divided into two equal portions, one of which was passed through a 1×10 cm. bed of Dowex 1-X10, 200-400 mesh, formate form ion-exchange resin to retain the organic acids, and the other portion was passed through a 1×5 cm. bed of Dowex 50-X10, 200-400 mesh, hydrogen form ion-exchange resin to retain the amino acids. The organic acids were removed from the Dowex 1 resin by gradient elution (8, 13), and the concentration of the individual acids was determined by titration with alkali following removal of the eluting acid. The amino acids were eluted from the Dowex 50 resin (9), and the concentration of the individual acids was determined following their separation by paper chromatography (11).

The protein nitrogen content of the lyophilized residues was determined by the micro Kjeldahl procedure (7).

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were extracted from the lyophilized residue by the differential perchloric acid procedure of Howard (6) and the phosphorus of each determined in the perchloric acid digests by the reduced molybdate method of Pons, *et al.* (10).

#### RESULTS AND DISCUSSION

The amounts in milligrams per plant of protein nitrogen, alcohol-soluble amino- and amide-nitrogen, RNA- and DNA-phosphorus, and

TABLE I  
EFFECT OF CUCUMBER MOSAIC VIRUS AND THIOURACIL TREATMENT ON  
SEVERAL COMPONENTS OF VAMORR 48 TOBACCO

| Treatment           | Time samples, hrs. | Protein N | Alcohol-soluble amino N | Alcohol-soluble amide N | RNA-P | DNA-P | Malic acid | Citric acid |
|---------------------|--------------------|-----------|-------------------------|-------------------------|-------|-------|------------|-------------|
| Mg./plant           |                    |           |                         |                         |       |       |            |             |
| Control             | 0                  | 10.0      | 0.32                    | 0.03                    | 0.37  | 0.05  | 8.04       | 3.83        |
|                     | 72                 | 21.0      | 0.60                    | 0.02                    | 0.64  | 0.06  | 15.90      | 3.61        |
|                     | 230                | 27.8      | 0.83                    | 0.17                    | 1.08  | 0.11  | 16.28      | 4.32        |
|                     | 286                | 38.6      | 1.49                    | 0.48                    | 0.74  | 0.16  | 20.10      | 4.69        |
| Thiouracil          | 72                 | —         | 0.82                    | 0.24                    | —     | —     | 14.68      | 3.34        |
|                     | 230                | 21.8      | 2.35                    | 1.15                    | 0.76  | 0.08  | 22.80      | 2.59        |
|                     | 286                | 20.6      | 4.18                    | 2.92                    | 0.22  | 0.04  | 23.25      | 1.77        |
| Virus               | 72(24)*            | —         | 0.54                    | 0.09                    | —     | —     | 15.79      | 4.88        |
|                     | 230(182)           | 27.0      | 0.86                    | 0.19                    | 1.17  | 0.11  | 14.90      | 3.54        |
|                     | 286(238)           | 26.0      | 0.95                    | 0.35                    | 0.75  | 0.18  | 18.96      | 2.58        |
| Thiouracil + virus  | 72(24)             | 19.6      | 0.66                    | 0.17                    | 0.66  | 0.06  | 11.20      | 2.86        |
|                     | 230(182)           | 14.8      | 1.76                    | 0.92                    | 0.28  | 0.04  | 13.34      | 1.70        |
|                     | 286(238)           | 18.7      | 3.40                    | 1.96                    | 0.15  | 0.07  | 21.36      | 1.04        |
| Mg./g. fresh weight |                    |           |                         |                         |       |       |            |             |
| Control             | 0                  | 2.0       | 0.06                    | 0.01                    | 0.07  | 0.01  | 1.16       | 0.77        |
|                     | 72                 | 2.4       | 0.07                    | 0.01                    | 0.07  | 0.01  | 1.79       | 0.41        |
|                     | 230                | 2.3       | 0.07                    | 0.01                    | 0.09  | 0.01  | 1.32       | 0.35        |
|                     | 286                | 2.3       | 0.09                    | 0.03                    | 0.04  | 0.01  | 1.73       | 0.28        |
| Thiouracil          | 72                 | —         | 0.13                    | 0.04                    | —     | —     | 2.36       | 0.54        |
|                     | 230                | 2.1       | 0.23                    | 0.11                    | 0.07  | 0.01  | 2.24       | 0.25        |
|                     | 286                | 2.1       | 0.44                    | 0.30                    | 0.02  | <0.01 | 2.44       | 0.18        |
| Virus               | 72(24)             | —         | 0.09                    | 0.02                    | —     | —     | 2.64       | 0.81        |
|                     | 230(182)           | 2.5       | 0.08                    | 0.02                    | 0.11  | 0.01  | 1.36       | 0.32        |
|                     | 286(238)           | 2.3       | 0.08                    | 0.03                    | 0.07  | 0.02  | 1.66       | 0.23        |
| Thiouracil + virus  | 72(24)             | 3.1       | 0.10                    | 0.03                    | 0.10  | 0.01  | 1.75       | 0.45        |
|                     | 230(182)           | 2.2       | 0.27                    | 0.14                    | 0.04  | 0.01  | 2.02       | 0.26        |
|                     | 286(238)           | 2.2       | 0.40                    | 0.23                    | 0.02  | 0.01  | 2.48       | 0.12        |

\* Hours after inoculation with CMV.

malic and citric acids in the experimental plants at the four sampling intervals are given in the upper part of Table I. The lower part of Table I presents the same data expressed in the conventional manner as milligrams per gram fresh weight. This is included only for purposes of comparison since the data are considered to be more meaningful when discussed on the basis of the amounts of the individual constituents per plant. The reasons for this are discussed elsewhere (12). The percentage change in the above constituents in the treated as compared with the control plants at the same time interval is presented in Table II. The values for protein



nitrogen and RNA- and DNA-phosphorus are missing from the thiouracil and virus treatments at 72 hours because undetermined portions of these dry residues were lost following extraction of the ethanol-soluble materials. It is probable that the amount of protein nitrogen was little affected by either virus or thiouracil alone at this interval since their combination, which caused the greatest reduction at 230 and 286 hours, resulted in only a slightly reduced level at 72 hours.

The control plants exhibited increased amounts of all measured components with increasing time except that the levels of lysine and phenylalanine in the alcohol-soluble fraction were lower at 286 hours than at 0

TABLE II  
CHANGES IN SEVERAL COMPONENTS INDUCED BY CUCUMBER MOSAIC VIRUS AND  
THIOURACIL EXPRESSED AS PERCENTAGE OF CONTROL  
AFTER TIME INTERVALS IN HOURS

| Component measured      | Thiouracil |     |     | Virus       |              |              | Thiouracil+virus |              |              |
|-------------------------|------------|-----|-----|-------------|--------------|--------------|------------------|--------------|--------------|
|                         | 72         | 230 | 286 | 72<br>(24)* | 230<br>(182) | 286<br>(238) | 72<br>(24)       | 230<br>(182) | 286<br>(238) |
| Protein-N               | —          | 78  | 53  | —           | 97           | 67           | 93               | 53           | 48           |
| Alcohol-soluble amino-N | 137        | 284 | 281 | 90          | 103          | 64           | 110              | 212          | 228          |
| Alcohol-soluble amide-N | 1200       | 677 | 608 | 450         | 111          | 73           | 850              | 535          | 408          |
| RNA-P                   | —          | 70  | 30  | —           | 108          | 101          | 103              | 26           | 20           |
| DNA-P                   | —          | 73  | 25  | —           | 100          | 113          | 100              | 36           | 44           |
| Malic acid              | 92         | 140 | 80  | 99          | 91           | 65           | 70               | 82           | 74           |
| Citric acid             | 92         | 60  | 38  | 130         | 81           | 55           | 81               | 40           | 22           |

\* Hours after inoculation with CMV.

hours (Table III). These amino acids reached their highest level at 230 hours and then decreased. There were reductions in the amounts of certain other constituents at some of the sampling intervals. Such decreases occurred in citric acid and the alcohol-soluble amide nitrogen (resulting from a lowered level of asparagine) between 0 and 72 hours and in certain of the individual alcohol-soluble amino acids, i.e., glycine and serine between 72 and 230 hours, and  $\gamma$ -aminobutyric acid, lysine, phenylalanine, threonine, tyrosine, and valine between 230 and 286 hours. There was a considerable decrease in the RNA-phosphorus between 230 and 286 hours. In addition, a sharp decrease in RNA-phosphorus during this same interval was exhibited by the virus-infected plants whose RNA concentration paralleled that of the control plants. The DNA-phosphorus increased in both the control and virus-infected plants during this interval. It is interesting that the control plants exhibited a maximum rate of protein production corresponding to the decrease in RNA. Increased levels of RNA are generally associated with net protein synthesis (4, pp. 226-295; 5), but exceptions have been noted (1, 2, 3).

TABLE III  
ALCOHOL-SOLUBLE AMINO ACIDS AND AMIDES, MILLIGRAMS PER PLANT AFTER TIME INTERVALS IN HOURS

|                              | Control |      |      |       | Thiouracil |       |       | Virus       |              |              | Thiouracil + virus |              |              |
|------------------------------|---------|------|------|-------|------------|-------|-------|-------------|--------------|--------------|--------------------|--------------|--------------|
|                              | 0       | 72   | 230  | 286   | 72         | 230   | 286   | 72<br>(24)* | 230<br>(182) | 286<br>(238) | 72<br>(24)         | 230<br>(182) | 286<br>(238) |
| Amino acids                  |         |      |      |       |            |       |       |             |              |              |                    |              |              |
| Alanine                      | 0.14    | 0.46 | 0.60 | 0.00  | 0.47       | 0.97  | 0.55  | 0.31        | 0.81         | 0.46         | 0.34               | 0.70         | 0.65         |
| $\gamma$ -Aminobutyric acid  | 0.10    | 0.24 | 0.68 | 0.46  | 0.17       | 0.80  | 0.40  | 0.15        | 0.76         | 0.28         | 0.16               | 0.02         | 0.59         |
| Arginine                     | 0.11    | 0.17 | 0.27 | 0.30  | 0.18       | 0.53  | 1.00  | 0.01        | 0.10         | 0.23         | 0.16               | 0.36         | 0.97         |
| Aspartic acid                | 0.20    | 0.33 | 0.38 | 0.65  | 0.38       | 0.50  | 1.01  | 0.41        | 0.36         | 0.37         | 0.46               | 0.53         | 1.04         |
| Glutamic acid                | 0.72    | 1.18 | 1.08 | 2.42  | 1.22       | 1.28  | 2.01  | 0.41        | 0.70         | 1.52         | 1.11               | 0.84         | 2.04         |
| Glycine                      | 0.03    | 0.53 | 0.97 | 1.06  | 0.40       | 0.35  | 0.23  | 0.37        | 0.68         | 0.36         | 0.20               | 0.17         | 0.25         |
| Leucine(s)                   | 0.20    | 0.22 | 0.32 | 0.41  | 0.18       | 0.47  | 0.40  | 0.02        | 0.36         | 0.21         | 0.15               | 0.34         | 0.45         |
| Lysine                       | 0.08    | 0.07 | 0.10 | 0.04  | 0.08       | 0.27  | 0.20  | 0.05        | 0.00         | 0.68         | 0.68               | 0.26         | 0.38         |
| Phenylalanine                | 0.30    | 0.48 | 0.56 | 0.25  | 0.45       | 0.80  | 0.54  | 0.41        | 0.88         | 0.35         | 0.37               | 0.41         | 0.94         |
| Proline                      | Tr**    | Tr   | Tr   | Tr    | 0.14       | 0.57  | 0.62  | 0.28        | Tr           | Tr           | 0.16               | 0.32         | 0.82         |
| Serine                       | 0.18    | 0.42 | 0.33 | 0.63  | 0.38       | 0.83  | 0.72  | 0.30        | 0.30         | 0.38         | 0.33               | 0.58         | 0.83         |
| Threonine                    | 0.20    | 0.20 | 0.42 | 0.31  | 0.22       | 0.81  | 0.83  | 0.14        | 0.33         | 0.21         | 0.21               | 0.60         | 0.90         |
| Tyrosine                     | 0.08    | 0.08 | 0.14 | 0.12  | 0.08       | 0.30  | 0.24  | 0.04        | 0.22         | 0.12         | 0.08               | 0.22         | 0.54         |
| Valine                       | 0.11    | 0.13 | 0.21 | 0.18  | 0.12       | 0.28  | 0.24  | 0.08        | 0.20         | 0.11         | 0.12               | 0.21         | 0.27         |
| Total amino acids            | 2.45    | 4.51 | 5.16 | 7.73  | 4.47       | 8.94  | 9.08  | 3.61        | 5.38         | 4.68         | 3.93               | 6.16         | 10.67        |
| Amides                       |         |      |      |       |            |       |       |             |              |              |                    |              |              |
| Asparagine                   | 0.17    | 0.10 | 0.74 | 1.06  | 0.15       | 1.46  | 4.82  | 0.05        | 1.17         | 0.72         | 0.21               | 2.67         | 4.57         |
| Glucamine                    | 0.14    | 0.14 | 0.94 | 3.78  | 2.30       | 10.42 | 25.05 | 0.89        | 0.72         | 2.86         | 1.52               | 6.65         | 15.46        |
| Total amides                 | 0.31    | 0.24 | 1.68 | 4.84  | 2.51       | 11.88 | 20.87 | 0.94        | 1.89         | 3.58         | 1.73               | 9.32         | 20.03        |
| Total amino acids and amides | 2.76    | 4.75 | 6.84 | 12.57 | 7.01       | 20.82 | 38.95 | 4.55        | 7.27         | 8.26         | 5.66               | 15.48        | 30.70        |

\* Hours after inoculation with CMV.

\*\* Trace.

Recent findings indicate that sub-units of RNA may play important roles in protein synthesis (5). Webster (14, 15) has shown that nucleotides specifically increase both the rate of amino acid incorporation into the protein and the protein content of the microsome-like particles derived from pea seedlings. It appears that ribonucleic compounds are formed during the synthesis of proteins, but the possibility exists that the molecules involved are smaller than RNA and also that there can be a turn-over of RNA without the occurrence of a net increase in this constituent (3). These findings might explain the increase in protein without a corresponding increase in RNA in the control plants in the present study. Nucleotides or their complexes with amino acids or peptides would have been extracted and discarded during the preliminary phases of the nucleic acid determinations. Such compounds are known to occur in tobacco,<sup>1</sup> but their relationship to nucleic acid and protein synthetic mechanisms has not yet been determined.

Earlier studies (12) indicated that CMV infection of Namor 48 tobacco produced an apparent stimulation of host metabolism characterized by increased concentrations of certain biochemical constituents at 168 hours after the initiation of infection. The present data show that, with the exception of malic and citric acids, the levels of all measured constituents are nearly equivalent in the control and virus-infected plants at 182 hours following inoculation. Virus infection caused a reduction in malic acid of about 10 per cent and in citric acid of about 20 per cent at this time. It appears that the stimulatory phase in the infected plants may be terminated near 180 hours after infection and is followed by considerable reductions, as compared to the control plants, in most constituents between this time and 238 hours after infection. The notable exception to this is RNA- and DNA-phosphorus. The amounts of these constituents in the infected plants were maintained throughout the experiment at levels equivalent to or greater than those of the control plants. Twenty-four hours after inoculation the infected plants showed a slight deficiency in amino-nitrogen, but a substantial increase of amide-nitrogen largely contributed by a high concentration of glutamine. This latter condition is of special interest because of its similarity to the occurrence of increased levels of glutamine in Turkish tobacco plants soon after infection with tobacco mosaic virus and at certain stages of infection with tobacco ringspot virus.<sup>2</sup>

The malate/citrate ratio increased in both the infected and control plants during the experiment. However, the ratio was higher in the infected plants 182 and 238 hours after infection. The increased ratio was due primarily to the relative decrease of citric acid since the increase in malic

<sup>1</sup> C. A. Porter and L. H. Weinstein, unpublished.

<sup>2</sup> C. A. Porter, unpublished.

acid was only slight. The same general pattern was followed by the two acids in thiouracil-treated plants, but the concentration changes were much greater.

Changes in amounts of the measured constituents of tobacco were similar in the thiouracil and combined thiouracil virus treatments although the magnitude of the differences was not the same in all cases. In general, if thiouracil and virus individually produced a change in the same direction relative to the control, their combination resulted in a greater change in that direction, but when they resulted in changes in opposite directions, their combination produced an effect intermediate between that of either alone. Thiouracil treatment singly or in combination with virus infection resulted in a decreased level of protein nitrogen, RNA- and DNA-phos-

TABLE IV

CHANGES IN AMIDE-NITROGEN AS PERCENTAGE OF AMINO-NITROGEN PLUS AMIDE-NITROGEN, AND IN GLUTAMINE- AND ASPARAGINE-NITROGEN AS PERCENTAGE OF AMIDE-NITROGEN AFTER TIME INTERVALS IN HOURS

|                                 | Control |    |     |     | Thiouracil |     |     | Virus       |              |              | Thiouracil + virus |              |              |
|---------------------------------|---------|----|-----|-----|------------|-----|-----|-------------|--------------|--------------|--------------------|--------------|--------------|
|                                 | 0       | 72 | 230 | 286 | 72         | 230 | 286 | 72<br>(24)* | 230<br>(182) | 286<br>(238) | 72<br>(24)         | 230<br>(182) | 286<br>(238) |
| Amide-N as % of amino + amide-N | 9       | 3  | 17  | 24  | 23         | 33  | 41  | 14          | 18           | 27           | 20                 | 34           | 37           |
| Glutamine as % of amide-N       | 42      | 56 | 52  | 76  | 94         | 87  | 83  | 94          | 36           | 78           | 87                 | 69           | 75           |
| Asparagine as % of amide-N      | 58      | 44 | 48  | 24  | 6          | 13  | 17  | 6           | 64           | 22           | 13                 | 31           | 25           |

\* Hours after inoculation with CMV.

phorus, and malic and citric acids, while producing increases in the alcohol-soluble amino- and amide-nitrogen pool. The total increase was contributed more by amino-nitrogen than amide-nitrogen, but, relative to the control, the percentage increase in amide-nitrogen was considerably greater than that of amino-nitrogen (Table II). The portion contributed by amide-nitrogen increased with time and was largely due to an increased level of glutamine, but asparagine also increased throughout (Table IV). The accumulation of glutamine is a further indication of a block in protein production at some point above the level of amino acid and amide formation. The increase in asparagine during the late stages of the experiment could indicate the occurrence of protein degradation. However, in the thiouracil-treated plants, the increase in amino- and amide-nitrogen between 230 and 286 hours was greater than the decrease observed in protein nitrogen (Table I) and consequently the increase in soluble nitrogen cannot be attributed entirely or even largely to proteolysis.

The malate/citrate ratio was increased considerably by thiouracil treatment. The increase was due primarily to a reduced concentration of citric



acid since malic acid increased with time in a manner parallel with the control but was maintained at a lower level than in the control. The observed changes in the malate/citrate ratio described for the various treatments will be discussed in detail in another communication dealing with the patterns of  $C^{14}O_2$  fixation in CMV-infected and noninfected tobacco grown in nutrient with and without thiouracil.

The results of the time-course studies presented here seem to further substantiate the earlier conclusion that thiouracil inhibits protein synthesis but does not materially impair the mechanism whereby the amino acids and amides are produced. The greatly reduced concentration of RNA resulting from thiouracil treatment is in accordance with the previous findings and is suggestive that protein synthesis may be decreased because of interference in nucleic acid and nucleotide metabolism by thiouracil. The unexpected decrease in the control plants of RNA during the time of maximum protein production does not alter the significance of the observed decrease resulting from thiouracil treatment because of the considerable difference in magnitude of the changes. However, this circumstance points out the need for further investigation to establish the exact relationship of nucleic acid metabolism to protein synthesis and the role played by smaller units such as nucleotides in these or allied processes of the tobacco plant.

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# THE SULFUR METABOLISM OF INSECTS. IV. THE CONVERSION OF INORGANIC SULFATE TO ORGANIC SULFUR COMPOUNDS IN COCKROACHES. THE ROLE OF INTRACELLULAR SYMBIONTS<sup>1</sup>

S. MARK HENRY AND RICHARD J. BLOCK<sup>2</sup>

## SUMMARY

The intermediary metabolism of inorganic sulfur into organic compounds in cockroaches was investigated by either feeding or injecting  $\text{Na}_2\text{S}^{35}\text{O}_4$  into *Blattella germanica* (L.) and *Periplaneta americana* (L.). The possible role of the gut microorganisms and the intracellular symbionts of *B. germanica* on the metabolic pathways was studied by determining the  $\text{S}^{35}$ -labeled compounds developed in normal insects and aseptically reared insects containing the symbionts and in cockroaches freed of symbionts.

In both normal and aseptic insects the  $\text{S}^{35}$  was found primarily in glutathione, cysteine, methionine, and methionine sulfoxide and only in limited quantities in taurine, sulfite, and several unidentified compounds. In symbiont-free *B. germanica* the  $\text{S}^{35}$  was detected only in sulfite and two unidentified compounds.

## INTRODUCTION

Available data summarized by Hilchey (13) on the dietary requirements of insects for methionine and cystine indicate that methionine is generally necessary and in some cases cystine is also required for growth and/or development. The only reported exception appears to be the German cockroach, *Blattella germanica* (L.). This insect does not need methionine and under proper conditions it will grow well in the absence of both cystine and methionine (12). It was found in this laboratory in 1955 that normal and aseptic cockroaches fed  $\text{Na}_2\text{S}^{35}\text{O}_4$  were able to synthesize methionine- $\text{S}^{35}$  and cystine- $\text{S}^{35}$  (14).

Some insects, including cockroaches, maintain an intimate association with certain microbial organisms in that the latter assume an intracellular location in distinct organs, the mycetomes (7, p. 42; 24, p. 190). In cockroaches, the mycetome is represented by large, discrete cells referred to as mycetocytes. These are distributed throughout the abdominal fat body (Fig. 1).

Blochmann in 1888 (1) described the intracellular microbes of cockroaches as symbiotic bacteria. Evidence that the relationship is mutualis-

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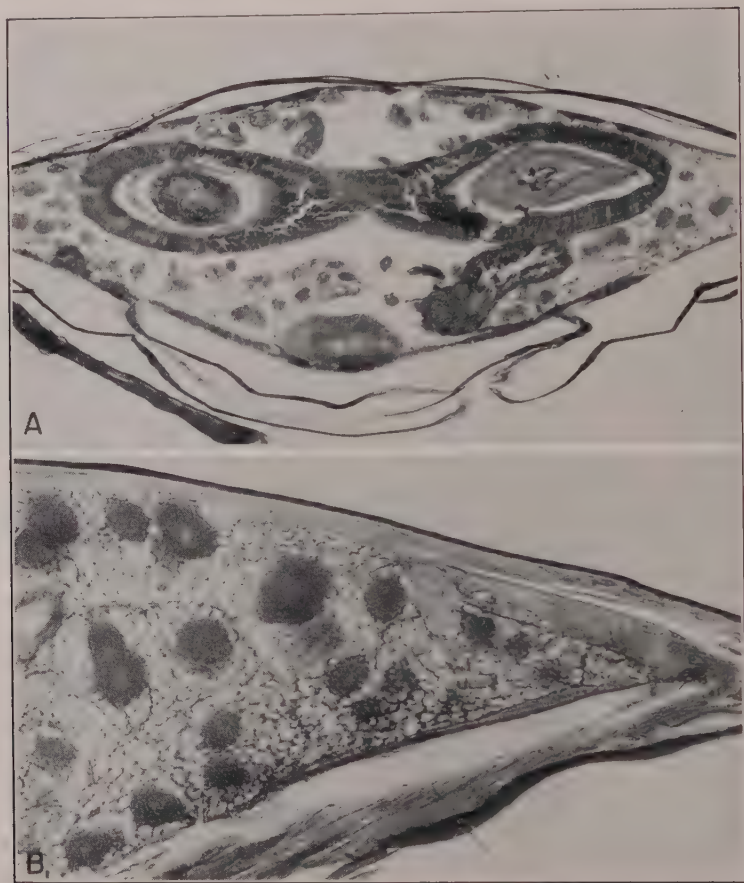


FIGURE 1. Abdominal cross section of a German cockroach stained with fast green, safranin, and crystal violet. (A) Gut is seen in two sections as a result of convolution ( $\times 85$ ). (B) Fat body with mycetocytes ( $\times 250$ ).

tic was presented by Brooks and Richards (5) who succeeded in rearing German cockroaches free of bacteroids. The symbiont-free cockroaches were highly abnormal but were able to survive and grow. Brooks postulated that the bacteroids of *Blattella* are involved in the growth rate, tanning of the cuticle, and in reproduction (4).

The present investigation was undertaken to determine the metabolic pathways involved in sulfate reduction and to ascertain if the extracellular and intracellular microbes participate in the ultimate conversion of sulfate to cystine and methionine.

#### EXPERIMENTAL

*Insects.* Nymphs of various ages as well as adults of *B. germanica* and *Periplaneta americana* (L.), maintained at  $24^{\circ}$ , were used throughout this investigation. As the findings on both species were similar no further dis-

tion will be made except where necessary. The majority of the experiments were carried out on normal insects containing both gut microorganisms and intracellular symbionts. Aseptic insects were reared as described previously (12) and symbiont-free German cockroaches were obtained by adding aureomycin to the diet at a concentration of 0.1 per cent (w/w) (5). This treatment resulted in destruction of the bacteroids inside growing eggs. Females with mature oöthecae were transferred to an aureomycin-free diet so that the aposymbiotic insects emerging from the oöthecae would at no time be in contact with the antibiotic. The aposymbiosis of the progeny of aureomycin-treated cockroaches was confirmed by the criteria of Brooks and Richards (5).

*Diet.* All insects were maintained on a mixture of dog biscuit (Gaines) 80 g.; dried brewers yeast (Nutritional Biochemicals Corporation) 10 g.; ethanol-insoluble liver concentrate (Liver Fraction "L," Nutritional Biochemicals Corporation) 5 g., and dextrose 5 g. (6).

*Methods.*  $\text{Na}_2\text{S}^{35}\text{O}_4$  was administered either by injection into the hemocele with a micrometer controlled syringe or by mixing it in the diet. At the end of the experimental period, the insects were homogenized with a Ten Broeck tissue grinder in about five times their weight of hot 80 per cent (v/v) ethanol or with 80 per cent ethanol containing 0.001 per cent (w/v) *N*-ethylmaleimide (9, 15) and washed three times with ten times their weight of 80 per cent (v/v) ethanol. The insoluble residue was dried and then hydrolyzed by refluxing for 16 hours with 3*N* HCl.<sup>3</sup> In several experiments the head and alimentary tract were removed from the insects and prepared separately for radiometric assay.

Ordinarily large quantities of labeled sulfate remained in the extracts and hydrolyzates. A large part of this was removed by adding an excess of carrier sulfate (1 ml. of 0.18*M*  $\text{H}_2\text{SO}_4$ ) and then precipitating the  $\text{SO}_4^{=}$  ion with an equimolar amount of  $\text{BaCl}_2$ . The supernatant liquid was evaporated to dryness in vacuo and the residue was dissolved in 10 per cent (v/v) 2-propanol.

The radioactive compounds were identified by means of one- and two-dimensional paper chromatography. The chromatograms were developed in the first direction with ethanol:tertiary butyl alcohol:58 per cent (w/v)  $\text{NH}_4\text{OH}:\text{H}_2\text{O}=60:20:5:15$  (v/v) (27) and in the second direction with tertiary butyl alcohol:88 per cent (w/v) formic acid: $\text{H}_2\text{O}=14:3:3$  (v/v) (2). The identities of the radioactive spots were confirmed where possible by co-chromatography with known compounds.

Labeled sulfite was identified by a modification of the technique of

<sup>3</sup> Hydrolysis with 6*N* HCl partially destroys methionine and other sulfur compounds. This destruction is markedly enhanced during hydrolysis in sealed ampoules at 15 pounds' pressure. When whole, unextracted cockroaches were hydrolyzed in the autoclave with 6*N* HCl a large proportion of the cysteine in glutathione was converted into cysteic acid. This oxidation of the cysteine to cysteic acid is largely or entirely prevented by the methods recommended.

Wilson and Bandurski (28). Five tubes were connected in series: tube 1 contained 5 ml. of 5 per cent (w/v)  $\text{CdCl}_2$  in 1 per cent (v/v) lactic acid (to purify nitrogen); tube 2, the cockroach homogenate plus a few mg. of Zn dust; tube 3, 10 mg. of  $\text{Na}_2\text{SO}_3$  in 5 ml. of  $\text{H}_2\text{O}$  plus Zn dust; tube 4, same as tube 1 (to trap mercaptans); and tube 5, 5 ml. of 0.1N KOH. Two or more cockroaches previously injected in the usual manner with  $\text{Na}_2\text{S}^{35}\text{O}_4$  were homogenized in 5 ml. of  $\text{H}_2\text{O}$  and quickly transferred to tube 2. One ml. of 6N HCl was added to the homogenate and to tube 3, after which the tubes were closed and immersed in boiling water for five minutes. Nitrogen was then bubbled through the tubes connected in series for 16 to 20 hours. The sulfur dioxide, trapped in the KOH solution, was oxidized with 0.5 ml. of 30 per cent (w/v)  $\text{H}_2\text{O}_2$ . After 15 minutes, 2.2 ml. of 6N HCl and 250 mg. of  $\text{BaCl}_2$  were also added to tube 5. The resulting precipitate of  $\text{BaSO}_4$  was washed several times with  $\text{H}_2\text{O}$ . Quantitative recovery was facilitated by using 1 to 2 ml. of 20 per cent (w/v) Chel 330<sup>4</sup> (diethylenetriamine pentaacetic acid) in hot 14 per cent (w/v)  $\text{NH}_4\text{OH}$  to dissolve the  $\text{BaSO}_4$ . The radioactivity of the chelated sulfate was then determined.

The  $\text{CdCl}_2$  solution in tube 4 was dried on a steam bath and the radioactivity of the residue was determined. No mercaptans were found.

Insects were prepared for cytological examination by fixing in Carnoy and Lebrun's fluid for 24 hours and then transferred to a solution of nitric acid in ethanol for two days (22). The tissue was dehydrated, embedded in Tissuemat and sectioned at 10 microns. Kodak AR-10 stripping film was applied to the sections as described by Taylor and McMaster (26). The slides were left in the dark for two weeks, developed in one-half strength Kodak C19b developer for three minutes, cleared, and dried.

## RESULTS

### METABOLISM OF $\text{Na}_2\text{S}^{35}\text{O}_4$ BY THE NORMAL COCKROACH

*Ethanol extracts.* An autoradiogram of the ethanol extract of cockroaches that had been injected with  $\text{Na}_2\text{S}^{35}\text{O}_4$  is reproduced in Figure 2 A. Twenty hours after injection, the principal radioactive spots are due to sulfate, glutathione, and an unknown (unknown number 1). Autoradiograms prepared from insects 44 hours after injection of  $\text{Na}_2\text{S}^{35}\text{O}_4$  are similar except that the sulfate streak appears to be separated into several components. The results of ten other experiments in which cockroaches were killed at different times after the feeding or injection of  $\text{Na}_2\text{S}^{35}\text{O}_4$  show that several other compounds appear shortly after unknown number 1 and glutathione. The most prominent, after the removal of excess sulfate, are methionine,

<sup>4</sup> Geigy Chemical Corporation, Ardsley, New York.

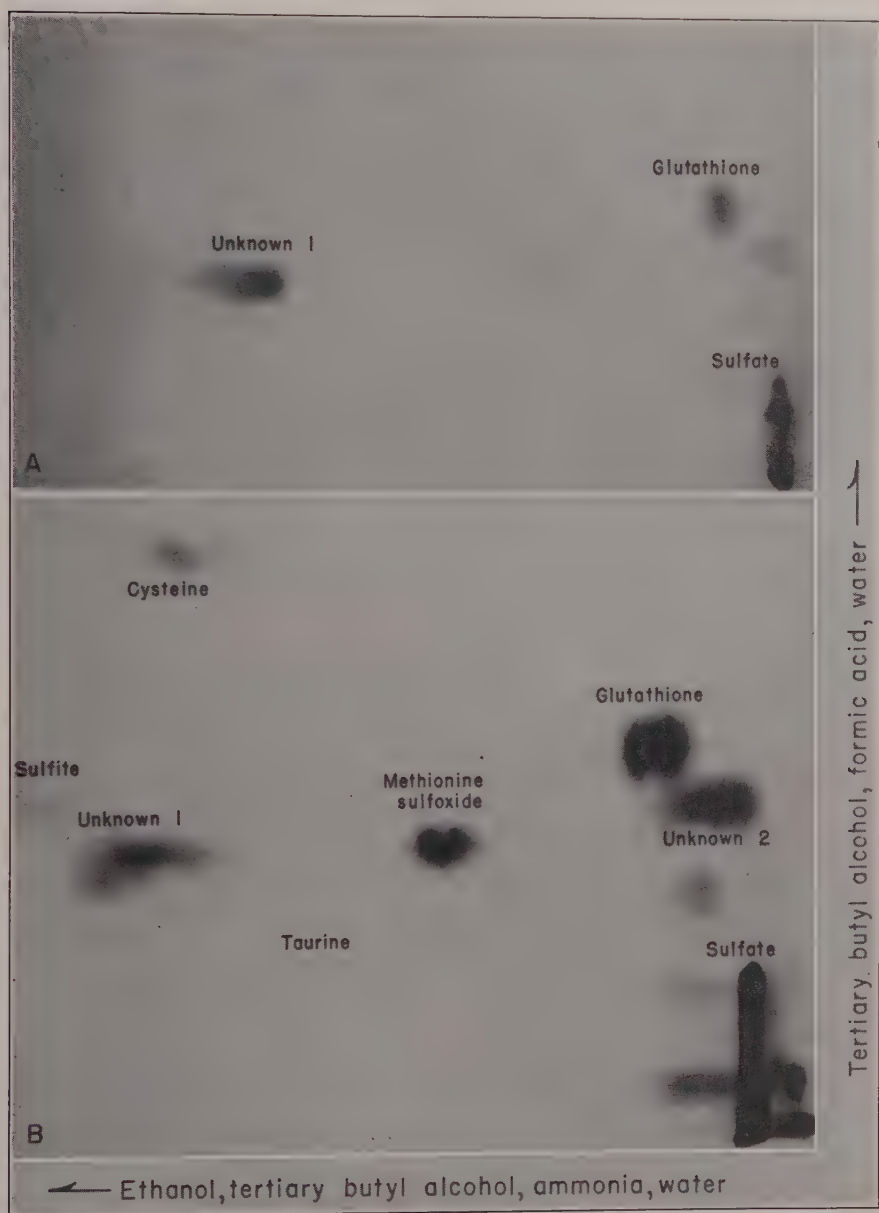


FIGURE 2. Autoradiogram of a chromatogram prepared from an extract of German cockroaches. (A) Injected with  $\text{Na}_2\text{S}^{35}\text{O}_4$  20 hours prior to extraction. (B) Fed for two weeks on  $\text{Na}_2\text{S}^{35}\text{O}_4$ .



methionine sulfoxide, and cysteine. Several minor radioactive compounds are seen near the chromatogram origin in the region of cystine, cystathionine, and cysteinesulfinic acid. A few autoradiograms also possess spots which have been tentatively identified as sulfite (stabilized with *N*-ethylmaleimide) and taurine.

Methionine- $S^{35}$  and cysteine- $S^{35}$  are not found in large quantities in the ethanolic extract. However, methionine sulfoxide- $S^{35}$  appears to accumulate in this fraction especially in cockroaches fed on  $\text{Na}_2\text{S}^{35}\text{O}_4$ .

Several additional radioactive compounds are found in extracts from which the excess sulfate was not removed. One of these, unknown 2 (Fig. 2 B), is seen only after extraction in the presence of *N*-ethylmaleimide. A sulfur-containing compound, the chromatographic position of which is identical to that of unknown 2, is obtained by placing glutathione-*N*-ethylmaleimide on the cation exchange resin, Dowex 50 ( $\text{H}^+$ ), and then eluting the material from the column with 0.4*N*  $\text{NH}_4\text{OH}$ .

Another acid-stable unknown, number 3, found occasionally in the extracts, does not react with *N*-ethylmaleimide and, like unknown 1, it is not retained by Dowex 50 ( $\text{H}^+$ ). The chromatographic position of unknown 3 is identical to that obtained with ammonium  $\beta$ -sulfolactate<sup>5</sup> in the solvent systems employed.

Although thiosulfate may be present, it has not been definitely detected chromatographically or colorimetrically (23).

*Acid hydrolyzates.* Hydrolyzates of the alcohol insoluble residues were found to contain labeled cystine, methionine, methionine sulfoxide, unknown number 1, and unknown number 3. When the insects were homogenized in the presence of *N*-ethylmaleimide, the hydrolyzates yielded very little cystine, but considerable quantities of cysteine and a compound, unknown 2 B, which is somewhat slower moving in the basic chromatographic solvent than unknown 2. A compound with identical chromatographic properties is obtained by boiling cysteine-*N*-ethylmaleimide with 3*N* HCl. In addition to being stable to acid hydrolysis, unknown numbers 1 and 3 behaved as anions on Dowex 50 ( $\text{H}^+$ ) while unknown 2 B is cationic.

Because most of the cystine and methionine in the cockroach is peptide-linked, the quantity of radioactivity in the cystine and methionine spots in the hydrolyzate is greater than in the extract. The ratio of labeled methionine sulfoxide to methionine is always greater than 4 in the acid hydrolyzates and about 1 in extracts of  $\text{Na}_2\text{S}^{35}\text{O}_4$ -injected cockroaches

<sup>5</sup>  $\beta$ -Sulfolactic acid was prepared as the barium salt by treating cysteic acid with  $\text{Ba}(\text{NO}_3)_2$  and  $\text{H}_2\text{SO}_4$ . After removing excess  $\text{HNO}_3$  under reduced pressure,  $\text{Ba}(\text{OH})_2$  was added and excess Ba was then precipitated with  $\text{CO}_2$ . Barium sulfolactate, precipitated from the supernatant by adding an equal volume of ethanol, was further purified by passage through a cation exchange resin (Dowex 50,  $\text{H}^+$ ).

but is greater than 15 in ethanol extracts of  $\text{Na}_2\text{S}^{35}\text{O}_4$ -fed cockroaches. In control experiments,  $\text{S}^{35}$ -labeled methionine was injected into heat-killed *B. germanica*. Extracts and hydrolyzates were prepared as with the sulfate-injected cockroaches. The ratio of methionine sulfoxide to methionine in these extracts and hydrolyzates was always less than 1.

*Reduction of sulfate to sulfite.* The results of several experiments conducted to determine whether sulfite is formed from sulfate injected into *Blattella* are given in Table I. Experiment A differs from the others in that the injected insects were homogenized in the presence of an excess of cold sulfite. In all the other experiments, the carrier sulfite was placed in tube 2

TABLE I  
REDUCTION OF  $\text{S}^{35}$  SULFATE TO SULFITE BY NORMAL GERMAN COCKROACHES

| Experiment | Method of $\text{Na}_2\text{S}^{35}\text{O}_4$ administration | Hours after injection* | $\text{SO}_2^-$ (as $\text{BaS}^{35}\text{O}_4$ ), c.p.m.** | Mercaptan fraction, c.p.m.* |
|------------|---------------------------------------------------------------|------------------------|-------------------------------------------------------------|-----------------------------|
| A          | Injected                                                      | 1                      | 293                                                         | 1                           |
|            |                                                               | 4                      | 507                                                         | 0                           |
| B          | Injected                                                      | 2                      | 35                                                          | 3                           |
|            |                                                               | 5                      | 212                                                         | 3                           |
|            | Added to dead insect homogenate                               | Control                | 1                                                           | 0                           |
| C          | Injected                                                      | 0                      | 18                                                          | 0                           |
|            |                                                               | 5                      | 246                                                         | 10                          |
| D          | Injected                                                      | 0                      | 26                                                          | —                           |
|            |                                                               | 5                      | 1863                                                        | —                           |

\*Samples taken immediately after injection are designated as 0 hours.

\*\*C.p.m. = counts per minute of an infinitely thick sample (total of recovered fraction).

as described in the experimental section. The effect of adding radioactive sulfate to killed (homogenized in hot ethanol) cockroaches is illustrated in experiment B, control.

*The distribution of  $\text{S}^{35}$  compounds in body tissues.* Attempts were made to ascertain whether the reduction of sulfate took place primarily in the gut or in the body of the insects. Therefore, the head together with the alimentary canal and its contents were removed at various times after injection and studied separately. The same radioactive materials were found in the carcass and in the excised sections with the former containing the larger proportion of  $\text{S}^{35}$  compounds. The dissections were, however, not entirely successful because in some cases much of the hindgut remained with the carcass and in other instances portions of the fat body adhered to the excised intestine.

In one experiment, a group of ten German cockroach nymphs were fed for one week on  $\text{Na}_2\text{S}^{35}\text{O}_4$ . Nine of these were extracted and hydrolyzed.



FIGURE 3. Microautoradiogram of an abdominal cross section of a German cockroach showing accumulation of radioactivity in integument, gut, and Malpighian tubules. Dark areas indicate heavy labeling.

All metabolites mentioned in the previous sections were found to be radioactive. The tenth insect was sectioned and microautoradiographs were prepared. The Malpighian tubules, midgut, hindgut, and epidermal layer of the integument were radioactive (Fig. 3). Less radioactive material remained localized in the mycetocytes.

#### METABOLISM OF $\text{Na}_2\text{S}^{35}\text{O}_4$ BY THE ASEPTIC COCKROACH

Five-week-old aseptically reared German cockroaches were fed  $\text{Na}_2\text{S}^{35}\text{O}_4$  for two weeks. Radioactive metabolites did not differ qualitatively from those of cockroaches reared under normal conditions.

TABLE II  
REDUCTION OF  $S^{35}$  SULFATE TO SULFITE BY APOSYMBIOTIC GERMAN COCKROACHES

| Experiment | Stage of insect    | Hours after injection | $SO_3^-$<br>(as $BaS^{35}O_4$ ),<br>c.p.m. |
|------------|--------------------|-----------------------|--------------------------------------------|
| E          | 3rd Instar nymphs  | 0                     | 18                                         |
|            |                    | 5                     | 246                                        |
| F          | Adults             | 0                     | 8                                          |
|            |                    | 5                     | 16                                         |
| G          | Adults             | 0                     | 14                                         |
|            |                    | 5                     | 41                                         |
|            | Last instar nymphs | 0                     | 27                                         |
|            |                    | 5                     | 90                                         |

#### METABOLISM OF $Na_2S^{35}O_4$ BY THE SYMBIOT-FREE COCKROACH

$Na_2S^{35}O_4$  was administered to aposymbiotic cockroaches either in the diet for 1 week or by injection. In contrast to the results obtained with normal and aseptic insects only  $S^{35}$  sulfite, unknown number 3, and occasionally unknown number 1 were found. Second and third generation aposymbiotic individuals maintained on a diet free of aureomycin failed to recover the ability to synthesize cystine and methionine from sulfate. The results in Table II suggest that more sulfate is reduced to sulfite by the younger insects.

#### DISCUSSION

The finding of Hilchey *et al.* (14) that normal German cockroaches are able to synthesize methionine and cystine from sulfate has been confirmed. Data presented in this paper suggest that sulfate is reduced to sulfite, possibly in organic combination with adenosine triphosphate (20) by normal, aseptic, and aposymbiotic cockroaches. The reduction of sulfate to sulfite appears to be a widespread phenomenon of living organisms as it has been observed in yeast (19) and bacteria (18) which are able to use inorganic sulfur to synthesize sulfur amino acids and in at least one vertebrate (8, 16). Aposymbiotic as well as normal cockroaches apparently are also able to fix the sulfite radical to an organic moiety. Fromageot (11) suggested that fixation of sulfite is mediated by acrylic acid stabilized by an enzyme. The principal end product of sulfate utilization in the chick embryo is taurine (16). Since  $SO_3^-$  and unknown 3 (possibly  $\beta$ -sulfolactic acid) but no taurine was produced from  $SO_4^-$  in the aposymbiotic cockroach, it is probable that a different mechanism of sulfite fixation occurs in the cockroach.

The fact that both normal and aseptic cockroaches are able to convert



sulfate-sulfur into methionine, methionine sulfoxide, glutathione, cysteine, taurine, etc. seems to indicate firstly that the usual gut commensals are not required for these transformations; that is, the microorganisms of the gut do not behave in the cockroach in a manner similar to the microorganisms of ruminants (3), and secondly that the presence of the intracellular symbionts is required. The question is still unresolved as to whether the symbionts *per se* convert sulfite and/or unknown 3 into methionine, cysteine, etc. or whether the aposymbiotic cockroach, as prepared for these experiments, is a pathological organism and consequently unable to effect the required transformations. Since aposymbiotic cockroaches maintained on an antibiotic-free diet for several generations fail to recover the ability to synthesize methionine and cystine from sulfate it is unlikely that aureomycin is acting directly upon any metabolic reaction(s). This problem is being further explored using reinfected cockroaches and aposymbiotic cockroaches obtained by several other methods.

If the assumption that the stable and unstable isotopes of sulfur are metabolically similar is correct, the presence of radioactive methionine, glutathione, etc. in the cockroaches after administration of  $S^{35}$  sulfate would suggest that the pathways proposed in the literature for the conversion of sulfate-S into organic S are also applicable to the normal and aseptic cockroach (10; 21, p. 342; 25). These schemes have been summarized by Margolis and Block (17). The unidentified compounds referred to as unknown 2 and unknown 2 B are probably not of any metabolic significance. The evidence indicates that both of these compounds are artifacts resulting from degradation of the *N*-ethylmaleimide adducts of glutathione and cytine, respectively, during preparation of the extracts and hydrolyzates.

Shortly after  $S^{35}O_4^{=}$  administration, glutathione is the most highly radioactive component in the cockroach. Labeled methionine and cysteine appear after glutathione. Although cysteine and methionine accumulate in the protein fraction, they are found in the alcoholic extract primarily as glutathione and methionine sulfoxide respectively. Methionine, once formed, is either incorporated into the proteins or oxidized. The presence of large amounts of methionine sulfoxide on chromatograms in these experiments raised the question of how much of this results from oxidation of methionine during the extraction, hydrolysis, and chromatographic procedures. Considering that a hydrolyzate of the protein residue remaining after ethanol extraction is subjected to strong acid and heat in addition to the same manipulations employed in preparing the ethanol extract, it should be expected that oxidation of methionine to the sulfoxide would be considerably greater in these fractions. In the case of  $S^{35}O_4^{=}$ -injected cockroaches which are extracted within 48 hours, this indeed appears to be true as indicated by the methionine sulfoxide-methionine

radioactivity ratios. However, when the insects are given  $S^{35}O_4^{=}$  in their diet over a period of one or two weeks, the ratios are reversed and methionine sulfoxide is considerably higher in the ethanol extracts than in the hydrolyzates. Both aseptic and normal cockroaches show these relationships. The low ratio of methionine sulfoxide to methionine obtained in control experiments with methionine- $S^{35}$  offers additional support to the belief that only a small amount of the observed methionine sulfoxide results from manipulation of the homogenate. The data suggest not only that the oxidation occurs in the insect but that methionine sulfoxide may be a storage depot for methionine (cf. 13) in the cockroach.

Although bacteria, yeast, and higher plants are able to utilize  $SO_4$  for the synthesis of methionine (cf. 14) this amino acid is not known to be a product of sulfate utilization in animals. It seems logical, therefore, to assume that the symbionts are involved in this conversion in the cockroach. Symbiont-free cockroaches are, however, able to reduce sulfate to sulfite and an unidentified compound, unknown number 3. Once the above substances are formed, however, further metabolism apparently ceases unless intracellular symbionts are present. This does not necessarily mean that methionine, glutathione, and other compounds cannot be synthesized by the aposymbiotic cockroach, for these may still be produced from cysteine, either supplied in the diet or synthesized from a precursor other than sulfate, sulfite, and unknown number 3.

Microautoradiograms (Fig. 3) of normal *Blattella* fed on  $Na_2S^{35}O_4$  do little to elucidate the role of the symbionts. The mycetocytes are not highly radioactive. The microautoradiograms, therefore, offer no evidence that the bacterioids participate in sulfate utilization. Since the greatest amount of radioactivity appears to be localized in the tissue of the gut and in the Malpighian tubules, perhaps the symbionts participate by supplying some enzyme or key intermediate. On the other hand, the mycetocytes could be the site of active transsulfuration, but the labeled sulfur compounds may have been extracted during the preparation of the specimen for autoradiography.

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# DISTRIBUTION STUDIES OF RADIOACTIVE FLUORINE-18 AND STABLE FLUORINE-19 IN TOMATO PLANTS<sup>1</sup>

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## SUMMARY

A study of distribution and site of accumulation of fluorine in tomato plants was carried out using NaF<sup>18</sup>, HF<sup>18</sup>, NaF<sup>19</sup>, and HF<sup>19</sup> applied as solutions through roots and cut vascular systems, and in gaseous form to the aerial parts. In order of decreasing concentration, the gross distribution of fluorine applied as NaF<sup>19</sup> through the soil was: roots, lower leaves, upper leaves, and stems. Regardless of the path of entrance of the fluorine into the plant, it was possible to wash up to 68 per cent of the fluorine from the leaves with distilled water and up to 83 per cent with water containing a detergent (Tween #20).

Distribution based on autoradiograms showed that most accumulation of fluorine-18 applied as HF<sup>18</sup> to tomato plants was at the tips and margins of leaflets and in the glands along the stem. Little fluorine-18 was found in the stems and petioles. In short-term or long-term experiments up to 80 per cent of the fluorine-18 or fluorine-19 of the leaves applied as HF was found in the stripped epidermis of *Sedum spectabile* Boreau. Distribution of fluorine-19 applied as HF<sup>19</sup> to tomato leaves after long-term accumulation was, in order of decreasing concentration: cell walls, chloroplasts, soluble proteins, mitochondria, and microsomes. With short-term accumulation of fluorine-18 applied as HF<sup>18</sup> the order was: soluble proteins, chloroplasts, cell walls, and mitochondria.

The fluorine in the chloroplasts was associated mainly with the proteinaceous substances since little or no fluorine-19 was found in the pigments and lipids.

## INTRODUCTION

It has been known for a long time that organic and inorganic substances taken up by underground and aerial portions of plants are translocated between the different organs (1; 5; 6; 15, pp. 876-890; 16). However, little information is available on the movement and sites of accumulation of fluorine in plants. A review of earlier work on the distribution of fluorine in the roots, stems, and fruits of various species grown under natural conditions can be found in the book by Bredemann (2, pp. 51-64). Recent information on gross distribution of fluorine in alfalfa, tomato, and celery exposed to HF<sup>19</sup> was obtained by Zimmerman and Hitchcock (23).

To gain more information on the distribution and site of accumulation of fluorine in plants, a number of experiments were initiated using tomato (*Lycopersicon esculentum* Mill.). Fluorine was applied as a sodium fluoride

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solution to the soil and cut stems or as hydrogen fluoride gas to the foliage through air. For this purpose radioactive sodium fluoride-18 ( $\text{NaF}^{18}$ ) and hydrogen fluoride-18 ( $\text{HF}^{18}$ ) as well as their stable equivalents were used. The study of penetration of  $\text{HF}^{18}$  and  $\text{HF}^{19}$  into leaves through the epidermis and leaching from the leaves was carried out using *Sedum spectabile* Boreau var. Brilliant from which the epidermis could be readily stripped from both surfaces of the leaves.

The pattern of distribution of fluorine was studied by making autoradiograms of fluorine-18 ( $\text{F}^{18}$ ) treated plants. Several attempts were made to locate the sites of accumulation of fluorine in the various components of leaf cells by microautoradiography of  $\text{F}^{18}$  containing material. However, the usual techniques used proved unsatisfactory, probably due to the high water solubility of the fluorides.

The term "fluorine" as used throughout this paper represents total fluorine content regardless of the types of fluorine compounds that may be present, except when radioactive measurements are made and then only  $\text{F}^{18}$  is measured.

#### MATERIALS AND METHODS

*Plants.* The plants used in these studies were tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) in the 5- to 8-leaf stage of development grown in 4-inch pots in composted soil containing 250 p.p.m. total fluorine, and *Sedum spectabile* Boreau var. Brilliant that had just completed a flowering cycle. The average fluorine content of leaves of nontreated plants of both species was 10 p.p.m.

*Source, application, and measurement of fluorine.* Fluorine was supplied to the soil as a 1 per cent solution of sodium fluoride added in increments of 50 ml. every two or three days, until the desired concentration was obtained in the various organs of the plant. Special care was taken to avoid contamination of the leaves with the sodium fluoride solution or with the treated soil.

Fumigation of plants with  $\text{HF}^{19}$  and the analyses of plant tissues and air for  $\text{F}^{19}$  were the same as used previously (3, 8, 9, 10, 11, 12, 13, 14, 23), the results for air analysis being expressed as parts per billion  $\text{HF}$  v/v [p.p.b. ( $10^{-9}$ )].

The experiments with  $\text{F}^{18}$  were carried out at Brookhaven National Laboratory,<sup>5</sup> Upton, New York, since the short half-life of  $\text{F}^{18}$  (112 minutes) and the millicurie quantities used in these experiments necessitated working near the source of production. This radioisotope is currently available at Brookhaven National Laboratory.

The  $\text{F}^{18}$  was produced by the reaction  $\text{O}^{16}(\text{t},\text{n})\text{F}^{18}$  using tritons obtained from the  $\text{Li}^6(\text{n},\text{t})\text{He}^4$  reaction.  $\text{Li}_2\text{CO}_3$  was irradiated in the thermal

<sup>5</sup> During this phase of the work the first two authors were Guest Associates of Brookhaven National Laboratory.

neutron flux at the Brookhaven reactor from which the  $F^{18}$  was separated by dissolving in dilute HCl and passing the solution through a column of chromatographic alumina. The  $F^{18}$  which was adsorbed on the alumina was eluted with 0.1M NaOH, and was supplied in the carrier-free form having radiochemical purity of better than 99.999 per cent (17, pp. 56-57; 19). One unit of  $F^{18}$ , which contained an average of 10 mc. of  $F^{18}$  in 30 ml. of solution, was used for each day's work 3.5 hours after removal from the reactor.

*Fumigation with  $F^{18}$ .* The fumigating cabinet used in these experiments is shown in Figure 1. The  $HF^{18}$  gas was obtained by adding  $NaF^{18}$  solution dropwise to a 98 per cent sulfuric acid solution contained in a polyethylene

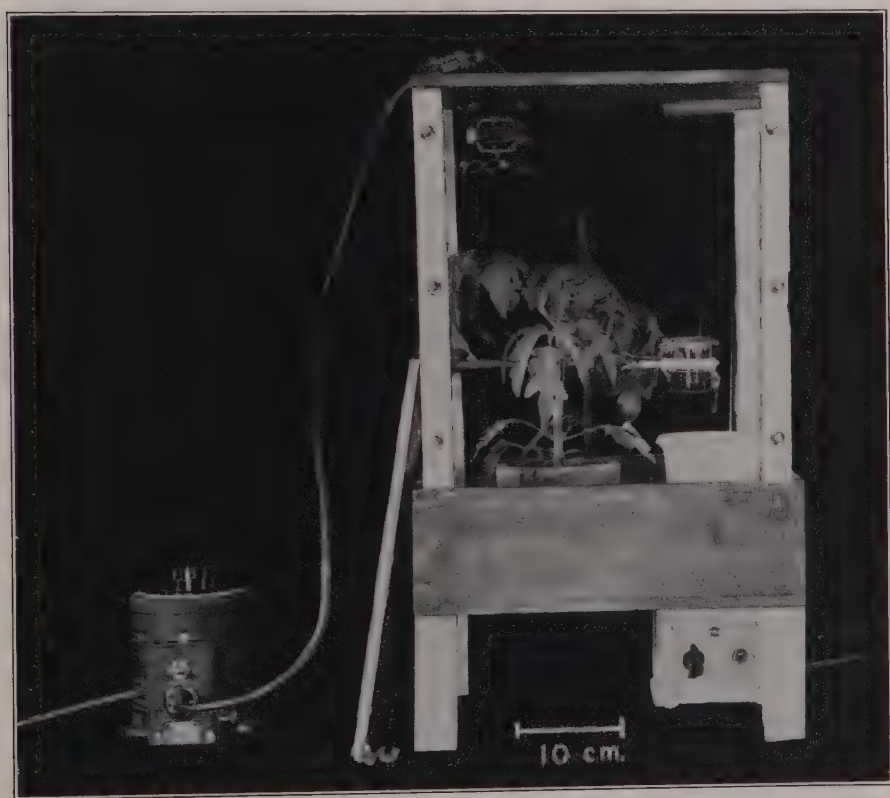


FIGURE 1. Cabinet used in fumigation with  $HF^{18}$ , made from a wooden frame coated with paraffin and provided with solid wooden bottom and top and Vinylite windows. The front frame is removable. The powerstat at left controls the electric fan attached at the top. The glass beaker containing the  $NaF^{18}$  solution is provided with a glass capillary siphon to deliver a constant rate of solution into the sulfuric acid contained in the polyethylene beaker below it, thus generating  $HF^{18}$ . The contents of this beaker are mixed by the magnetic stirrer at lower right. The treatment of the plant placed in the cabinet is started after the door has been attached to the cabinet.



beaker of 600-ml. capacity provided with a magnetic stirrer. A constant rate of dropping was obtained by using glass capillary siphons with a delivery rate of approximately one drop every two seconds. The fumigation time varied from one-half to two hours. The air within the cabinet was continuously circulated by a small electric fan. The plants were illuminated by two 125-watt electric bulbs, external to the cabinet. To avoid contamination of the working space with the  $\text{HF}^{18}$ , the cabinet was placed in a ventilated hood during fumigation.

Several experiments were made to detect the evolution of fluorine from inert surfaces and plant material treated with either  $\text{F}^{19}$  or  $\text{F}^{18}$ . When  $\text{F}^{18}$  was applied, as shown in Figure 1, the following technique was used. The material was placed in a closed polyethylene bag, approximately 200  $\times$  400 mm., provided with polyethylene inlet and outlet tubes. The outlet was connected through a short Tygon tube to a glass U-tube filled with a mixture of activated charcoal, activated alumina and molecular sieve in equal proportions, held by glass wool plugs (Fig. 2). The U-tube was cooled to  $-79^\circ \text{C}$ . in a dry ice-trichloroethylene bath. The other end of the U-tube was connected through an oil-filled bubble counter to a water pump, the sweeping rate not exceeding three bubbles per second. After the completion of the absorption, the contents of the U-tube were transferred to a counting tube and the radioactivity measured. When plant material was used, the cut stem was placed in water and an externally located light bulb of 125 watts was used to illuminate it.

*Counting methods.* For measurement of radioactivity, samples were placed in 10-ml. cellulose nitrate counting tubes (12  $\times$  100 mm.). The detector was a Harshaw well crystal of sodium iodide (TI) measuring 50  $\times$  44 mm. with a well 37  $\times$  19 mm. in which a copper liner was placed. The maximum useful volume with this detector is 3 ml. The counting error using an Atomic Model 1050A power supply and scaler did not exceed 1 per cent at the level of 1,000 counts per minute and 3 per cent at 500,000 counts per minute. Most of the material was measured within the range of 1,000 to 600,000 counts per minute. The background usually varied from 170 to 200 counts per minute.

Since the decay products of  $\text{F}^{18}$  are a single 0.56 Mev positron, with two coincident 0.51 Mev annihilation gamma rays and the inert gas, neon, no adverse effects could be expected on the plants from this treatment at the concentrations used. The concentration of  $\text{HF}^{18}$  in the atmosphere of the cabinet was estimated to be less than 0.01 p.p.b. v/v.

*Autoradiography.* Autoradiograms were made by placing the intact treated material on medical X-ray film which was protected by covering the emulsion with a Mylar film, less than 10  $\mu$  in thickness. These were stored in X-ray exposure holders until the film was removed for processing at least 18 hours later.



FIGURE 2. Equipment used to wash with air and sample the gas passed over treated material contained in the polyethylene bag which in this case is a tomato plant with its cut stem dipped in a beaker of water. The bag is provided with an air inlet on the lower right and a polyethylene outlet on the upper left connected by a short Tygon tube to a glass U-tube containing the absorbing material.

*Fractionation of plant material.* Various components of the leaves were obtained by suspending and homogenizing them in 0.5 *M* sucrose solution at 0° to 10° C. Cell walls, chloroplasts, mitochondria, microsomes, soluble proteins, and other solutes were separated from one another by fractional centrifugation. A Servall Omnimixer with a 400-ml. stainless steel container was used in the first operation. Approximately 10 grams of fresh leaves were homogenized in 40 ml. of 0.5 *M* sucrose solution for three minutes at a speed given by 40 volts. The suspension was gravity filtered through a Nitex #35 sieve with rectangular openings of 35  $\mu$ . The residue on the filter was suspended, ground, and filtered twice more under the same conditions. The last sediment on the filter obtained after the third homogenization constituted the cell wall fraction and was light green in color.

The combined filtrates were centrifuged at 3,000 $\times$ G using a Servall angle centrifuge for 10 minutes, and the dark green sediment of chloroplasts was collected. Before sedimenting mitochondrial and microsomal fractions, the filtrate was centrifuged at 5,000 $\times$ G for 10 minutes and the greenish precipitate was discarded. The mitochondria, and possibly some microsomes, were then separated by centrifuging the remaining supernatant at 34,000 $\times$ G for 20 minutes. If both mitochondria and microsomes were to be separated from the above suspension, the mitochondria was sedimented first at 10,000 $\times$ G for 20 minutes and then the microsomes were sedimented at 80,000 $\times$ G for 2 hours, a Spinco Ultracentrifuge being used for the latter operation. The supernatant was boiled after adding an equal volume of ethanol, thus precipitating the soluble protein. The supernatant containing the soluble fluorine was assayed only when F<sup>18</sup> was used in the experiments since the large amount of sugar in the solutions precluded the determination of F<sup>19</sup> by standard chemical procedures.

The homogeneity of the above separated fractions was established by microscopic examination for cell wall and chloroplast material. For the mitochondrial and microsomal fractions no conclusive evidence could be obtained from optical microscopy. However, the mitochondrial fraction sedimented at a speed from which mitochondria would be expected to be separated (21), and this fraction showed an affinity for Janus green B stain (4). The microsomal fraction was characterized only by the centrifugal force used in its separation. A protein content of 56 per cent was found in the supernatant fraction when it was boiled down in 50 per cent alcohol after the final centrifugation.

When experiments were carried out to determine foliar penetration of HF<sup>18</sup> or HF<sup>19</sup>, the epidermis was stripped from the mesophyll of a leaf of *S. spectabile* with a scalpel and forceps. The usual techniques and precautions were observed in handling the radioactive F<sup>18</sup> material, the working space being continuously monitored with a Geiger counter.

## RESULTS AND DISCUSSION

*Gross distribution.* In initial experiments  $\text{NaF}^{19}$  was administered through the soil in solution and was determined in the major organs of tomato plants. After the first darkening at the margins appeared on the leaves, the plants were harvested and divided into roots, stems, and leaves. They were then dried, milled, and analyzed separately for fluorine, showing the following content: roots 731, stems 65, and leaves 414 p.p.m. F.

In another experiment the various leaves were harvested separately to determine whether the concentration of fluorine varied from the base to the upper region of a plant that contained eight expanded leaves, and

TABLE I

DISTRIBUTION OF FLUORINE IN TOMATO LEAVES ACCORDING TO THEIR POSITION ON THE PLANT AFTER ADDING  $\text{NaF}^{19}$  TO THE SOIL

| Position of the leaf      | Fluorine concn.<br>in p.p.m. |
|---------------------------|------------------------------|
| Two lower leaves          | 198                          |
| Third and fourth leaves   | 84                           |
| Fifth and sixth leaves    | 72                           |
| Seventh and eighth leaves | 51                           |
| Unexpanded leaves at tip  | 23                           |

results are shown in Table I. The first evidence of markings occurred on the fifth, sixth, and seventh leaves, although the lower leaves contained much more fluorine. These results are similar to those reported previously for naturally-occurring fluorine by others (2, Table 10, pp. 53-54; 22) and were also obtained by Zimmerman and Hitchcock (23, Table VII, p. 278).

The markings sometimes appear on the leaflets on one side of a leaf while leaflets on the other side show no such markings. The marked and uninjured leaflets from opposite sides of the leaf were harvested and analyzed separately. Marked leaflets had 195 p.p.m. of fluorine, and the unmarked leaflets had 101 p.p.m. of fluorine.

A similar unequal distribution of soluble materials was also found when the vascular system of a tomato plant was stained by cutting off a leaf on one side of the plant and inserting the petiole in a 1 per cent solution of acid fuchsin. The fuchsin was drawn into the vascular tissues connected to this petiole. When the plants were cleared of chlorophyll, the staining in the vascular system showed very clearly that fuchsin from this petiole went into one side but not the other of leaves above the site of entrance. When a petiole on the opposite side of the plant was inserted in crystal violet or a fluorescent dye, such as fluorescein (Fig. 3), the material moved upward in other tracheal tubes, some of which fed the other





FIGURE 3. Fluorescence of a tomato plant taken under filtered ultraviolet light after introducing fluorescein through the petiole. (A) Entire plant showing the petiole through which the 0.1 per cent aqueous solution of fluorescein was introduced at lower center, the fluorescein being taken up only on the side of the plant connected to the vascular system associated with this petiole. (B) Details of the leaf on the right of A, showing the clear-cut separation between the portions of the leaf connected to different vascular strands.

half of the leaf partially stained with fuchsin. By this simple differential staining procedure it was possible to demonstrate that materials move through separate tracheal tubes, and these branch so they feed into different lateral sections of the leaf. In a similar experiment a downward movement of acid fuchsin into the root, as well as the upward movement, was observed.

*Autoradiograms.* Autoradiographic experiments with plants fed  $\text{NaF}^{18}$  through the petiole produced a similar pattern of distribution as shown by Figure 4, except that the fluorine was found predominately in the margin and at the tips of the leaflets, and not in the vascular system through which it presumably passed.

A tomato plant treated with gaseous  $\text{HF}^{18}$ , in a 2-hour experiment, showed a decrease in the concentration of  $\text{F}^{18}$  in the leaves, from the base of the plant to the top (Fig. 5). In this particular experiment the plant was isolated from the  $\text{HF}$  gas by two layers of Parafilm tied just above the cotyledons. These results suggest a possible translocation of fluorine from the leaves downward to the roots.

To test this possibility a potted tomato plant was fumigated for a longer period of 3 hours. In this case the pot was isolated from the  $\text{HF}$  gas by enclosing it within a bag made of two layers of polyethylene. During the fumigation a continuous stream of air was blown through the bag at low positive pressure to avoid the possibility of contamination from the  $\text{HF}$  gas. After the fumigation samples of soil from the surface and below had no radioactivity, indicating that the enclosed pot was free of contamination from the  $\text{HF}$  gas. However, the isolated roots had a radioactivity of 23 counts per minute per milligram of dry material, showing that a translocation of  $\text{F}^{18}$  took place from the leaves to the roots.

Another experiment with  $\text{HF}^{18}$  was carried out to determine the site of accumulation of fluorine in more detail. In this case the aerial portion of the plant was exposed to gaseous  $\text{HF}^{18}$  for one hour. After this fumigation the soil was carefully washed from the roots, and the roots were dried with absorbent towels so the whole plant could be autoradiographed (Fig. 6 A and B). An accumulation took place here along the margins and especially at the tips of leaflets similar to the distribution when the plant was fed  $\text{NaF}^{18}$  through the petiole (Fig. 4). In addition, fluorine is found associated with some veinlets and in other small discrete areas especially along the stem (Fig. 6 C and D); these small areas of high fluorine concentration probably were glandular trichomes.

*Leaching and leaf penetration.* It has been known for a long time that plants can absorb and lose substances through their leaves. A recent paper (20) reviewed the earlier work on this subject and reported studies of this phenomenon using radioactive isotopes. These studies were carried out by leaching with water the above-ground parts of plants treated through the

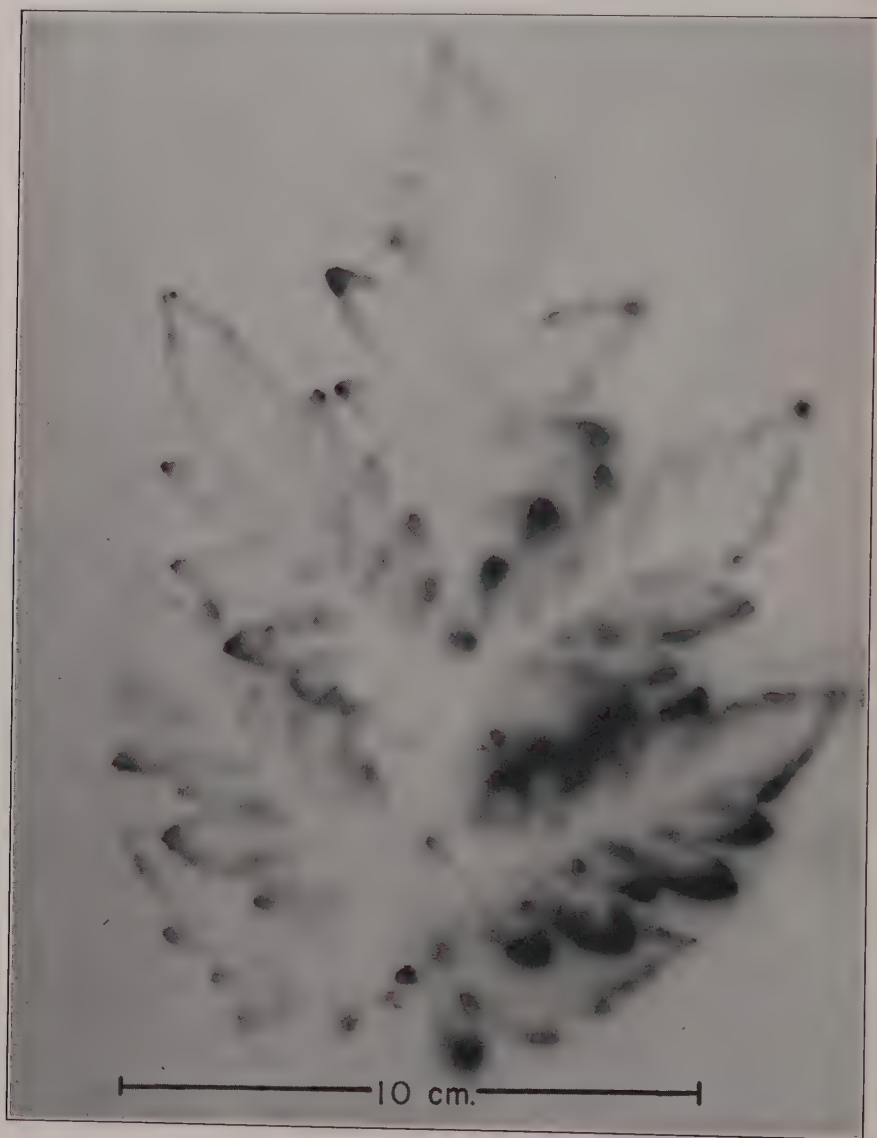


FIGURE 4. Autoradiogram of tomato leaf from a plant fed through a petiole with  $NaF^{18}$  solution at pH 7, showing an unequal distribution of  $F^{18}$  and a concentration along the margins, especially at the tips of leaflet segments ( $\times 0.75$ ).

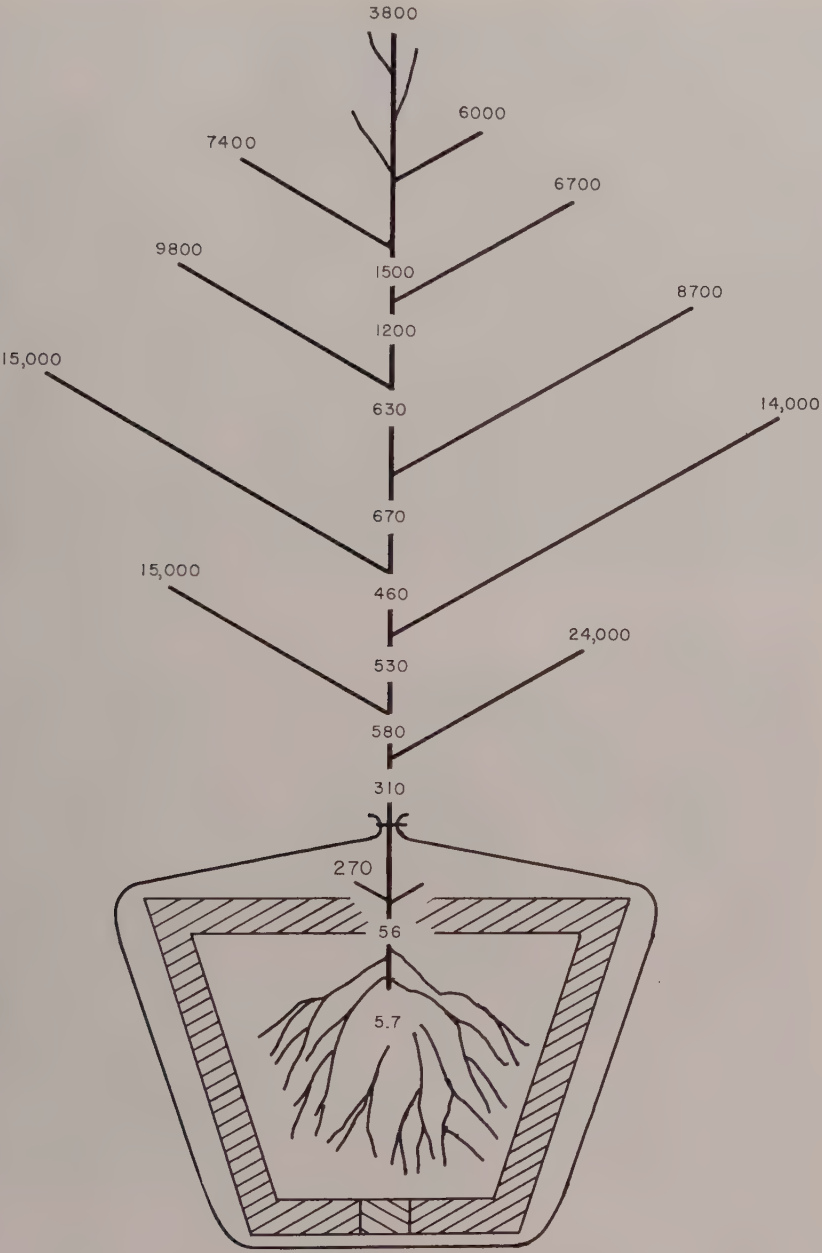


FIGURE 5. Diagram of a tomato plant exposed to gaseous  $\text{HF}^{18}$ . The numbers represent the counts per minute per milligram of dry material found in the various parts of the plant. The pot and lower portion of the stem were isolated from the  $\text{HF}^{18}$  gas.





FIGURE 6. Tomato plant treated with  $HF^{18}$  gas and its autoradiogram. (A) The pressed and dried tomato plant. (B) Autoradiogram of the above plant indicating the presence of  $F^{18}$  in highest concentration in leaf blades, intermediate in the stem and petioles, and lowest in the roots. (C) Details of B, showing the high concentration of  $F^{18}$  along the margins of the leaflets with some activity along the veinlets. (D) Details of B, showing small points of high concentration along the stem, probably associated with the glandular trichomes.

roots with various radioisotopes and determining the radioactivity of the leachate. In all cases losses were observed, the older leaves showing more loss than the younger ones.

Since the uptake and loss of fluorine from plant leaves had not been previously reported, a study was undertaken to determine the penetration and leachability of  $F^{19}$  and  $F^{18}$ .

The penetration of  $HF^{18}$  into leaves was studied by fumigating *S. spectabile* with  $HF^{18}$  for one-half hour and stripping the epidermis from two leaves immediately after fumigation and one leaf 3 hours after fumigation. The epidermis and mesophyll were analyzed for  $F^{18}$  separately (Table II). At this early stage it can be seen that up to 80 per cent of the fluorine in the leaf is on and within the epidermis, the remainder being

TABLE II  
DISTRIBUTION OF  $F^{18}$  IN THE LEAVES OF *SEDUM SPECTABILE*  
FUMIGATED FOR ONE-HALF HOUR

| Leaf tissue | Per cent of $F^{18}$         |                              |
|-------------|------------------------------|------------------------------|
|             | Immediately after fumigation | Three hours after fumigation |
| Epidermis   | 76                           | 82                           |
| Mesophyll   | 24                           | 18                           |

found in the mesophyll. Essentially the same distribution was obtained 3 hours after the plant was removed from the fumigation cabinet.

In another experiment *S. spectabile* was fumigated continuously for one week with an average concentration of 10 p.p.b. v/v  $HF^{19}$ . At the end of this fumigation 9 leaves were completely stripped of epidermis, and the epidermis and mesophyll were analyzed separately. A similar set of 9 leaves with epidermis intact was leached by dipping 10 times successively in each of three polyethylene beakers containing 100 ml. of water after sealing the cut petiole with melted Tissuemat to avoid loss from the sap exuding from the cut surface. The leaves were stripped as above and the wash water, epidermis, and mesophyll analyzed separately for  $F^{19}$ . To see if an exudation of fluorine will occur after the first washing, the *S. spectabile* plant was washed thoroughly and left in the greenhouse for one week following the fumigation when the leaves were leached as described above. At this time the plant was washed again and two weeks after fumigation a similar sample was leached and analyzed. Table III presents these results. As can be seen, the distribution of  $F^{19}$  in the epidermis and mesophyll of unwashed leaves was similar to that found in the short-term experiment described in Table II. In addition to this, the leaching experiments showed that 30 per cent of the  $F^{19}$  was leached from the leaves immedi-

TABLE III

DISTRIBUTION OF  $F^{19}$  IN LEAVES OF *SEDUM SPECTABILE* AND IN THE LEACHATE OF THE FUMIGATED LEAVES AFTER EXPOSURE OF ONE WEEK AT 10 P.P.B. v/v  $HF^{19}$

| Material analyzed | Percentage $F^{19}$ in leaf tissue and leachate |                              |                            |                              |
|-------------------|-------------------------------------------------|------------------------------|----------------------------|------------------------------|
|                   | In nonwashed leaves                             | Immediately after fumigation | One week after fumigation* | Two weeks after fumigation** |
| Epidermis         | 73                                              | 48                           | { 80<br>20                 | { 92<br>8                    |
| Mesophyll         | 27                                              | 22                           |                            |                              |
| Leachate          | —                                               | 30                           |                            |                              |

\* These leaves were washed a week previous to this final washing.

\*\* These leaves were washed twice the two weeks previous to this final washing.

ately after fumigation (Table III), 20 per cent one week later, and 8 per cent after two weeks. These results with  $F^{19}$  and  $F^{18}$  suggest that rather large amounts of fluorine might be removed from leaves when they are exposed to rain or other washing action (2, p. 145). The leached fluorine falls on the soil where, in these low concentrations, it is converted into insoluble forms unavailable to the plants as was demonstrated by MacIntire (7).

In order to determine the rate of washing, an experiment was carried out with an  $HF^{18}$  fumigated plant in which the same leaf, while on the plant, was washed by dipping successively 3 times in each of 6 beakers, each containing 100 ml. of distilled water. The same leaf was also washed one hour later using the same procedure, and the results are shown in Table IV. It can be seen that the leaf has the ability to form a new amount of washable fluorine, these results being in agreement with those shown in Table III. A second experiment was carried out using distilled water to

TABLE IV

PERCENTAGE DISTRIBUTION OF  $F^{18}$  FOUND IN THE LEACHATES OF THE SAME INTACT TOMATO LEAF WASHED WITH WATER OR WITH WATER CONTAINING TWEEN #20

| Number of washings                | Percentage of $F^{18}$ in leachate from the leaf according to method of washing |                        |                                                 |
|-----------------------------------|---------------------------------------------------------------------------------|------------------------|-------------------------------------------------|
|                                   | Water                                                                           |                        | Water containing Tween #20 (1 drop per 100 ml.) |
|                                   | Immediately after fumigation                                                    | Same leaf 1 hour later |                                                 |
| 1                                 | 47.4                                                                            | 3.9                    | 61.5                                            |
| 2                                 | 6.4                                                                             | 1.1                    | 7.2                                             |
| 3                                 | 2.9                                                                             | 0.8                    | 4.9                                             |
| 4                                 | 2.0                                                                             | 0.5                    | 4.7                                             |
| 5                                 | 1.5                                                                             | 0.4                    | 2.9                                             |
| 6                                 | 1.5                                                                             | 0.4                    | 1.9                                             |
| Total percentage washed from leaf |                                                                                 | 68.8                   | 83.1                                            |

which Tween #20 was added, showing that more fluorine could be washed from the leaf than when water alone was used.

In another experiment  $\text{NaF}^{18}$  at pH 7 was introduced into a tomato plant by dipping the cut stem into the solution. When the leaves were washed as above, 45 per cent of the total fluorine was removed.

*Evolution of volatile fluorine from leaves and inert surfaces.* The above experiments show that a large portion of fluorine taken up by leaves or through stems is lost from a plant by washing with water. It seemed of interest to determine if the plant loses some of its fluorine to the atmosphere (2, p. 158; 18, p. 308). For this purpose 100 tomato plants were fumigated for 10 days with an average concentration of 19 p.p.b. v/v of  $\text{HF}^{19}$ , after which the plants were transferred to a similar cabinet in which the natural air contained less than 0.5 p.p.b. v/v of  $\text{HF}^{19}$ . After 24 hours during which scrubbed air was passed through the cabinet at the rate of one exchange per minute, an analysis for volatile fluorine was made by sampling the air for 24 hours through an absorber at a bubbling rate of 40 cubic feet per hour in still air. The amount of fluorine found in the analysis did not exceed the concentrations usually found in this cabinet, indicating the absence of a measurable quantity of fluorine given off by the plants in a form ionizable in water. However, this experiment does not exclude the possibility of an emission of fluorine containing volatile substances in nonionized form, such as  $\text{CF}_4$ ,  $\text{CH}_3\text{F}$ , etc. A similar short-time experiment was carried out in which a tomato plant was treated through the stem for 2 hours with  $\text{NaF}^{18}$  at pH 7 and afterwards placed in a plastic bag through which air was passed at a slow rate for 1 hour (Fig. 2). The analysis of the absorbing material in the U-tube showed no radioactivity, indicating the absence of any evolution of  $\text{F}^{18}$ . However, it should be noted that this experiment is conclusive only for the short time used, further possible metabolic action not being excluded.

Another tomato plant was treated with gaseous  $\text{HF}^{18}$  for a 2-hour period, placed in a similar plastic bag, and the absorbing material was analyzed after the air was passed through the bag for 2 hours. In this case an activity of 112 counts per minute was obtained. A fraction of a leaflet ( $5 \times 5$  mm.) showed an activity greater than the counting capacity of the scaler (over 600,000 counts per minute). The slight activity obtained in the absorbing tube probably came from surface contamination rather than from within the plant. To test this hypothesis a plastic surface of Vinylite simulating a tomato plant was fumigated with  $\text{HF}^{18}$ , placed in the plastic bag, and the washing air analyzed as for the previously described tomato plant. In this case a relatively high activity of over 28,000 counts per minute was found, thus confirming the hypothesis of surface contamination. When the plastic surface was washed with water, only 56 per cent of the fluorine could be removed.



Since Vinylite showed an ability to retain  $\text{HF}^{18}$ , an experiment was carried out with an inert material, polytetrafluoroethylene (Teflon). Equal surfaces of this material were cut into portions  $7 \times 15 \times 1$  mm. and exposed for 3 hours to  $\text{HF}^{18}$ . The activity of the exposed chips of Teflon measured before and after washing with several solutions showed that the capacity of the solvent for removing F from the chips increased in the order: acetone, 16 per cent; water, 58 per cent; 2.5 per cent  $\text{NH}_4\text{OH}$ , 60 per cent; 2N HCl, 86 per cent; and 1 per cent Alkanox, 86 per cent. From these

TABLE V  
DISTRIBUTION OF  $\text{F}^{19}$  AND  $\text{F}^{18}$  IN THE VARIOUS CELLULAR CONSTITUENTS OF  
TOMATO LEAVES TREATED THROUGH THE AERIAL PORTION OF THE PLANT

| Cellular constituent | $\text{F}^{19}$ , p.p.m. dry weight<br>at the end of 10 days'<br>fumigation | $\text{F}^{18}$ , counts/min./mg.<br>dry weight after 2<br>hours' fumigation |        |
|----------------------|-----------------------------------------------------------------------------|------------------------------------------------------------------------------|--------|
|                      |                                                                             | Nonwashed                                                                    | Washed |
| Cell walls           | 1,685                                                                       | 142                                                                          | 155    |
| Chloroplasts         | 693                                                                         | 176                                                                          | 145    |
| Mitochondria         | 99                                                                          | 78                                                                           | 34     |
| Microsomes           | 21                                                                          | —                                                                            | —      |
| Soluble proteins     | 371                                                                         | 592                                                                          | 662    |
| Supernatant          | —                                                                           | 82*                                                                          | 37*    |

\* The concentration of  $\text{F}^{18}$  is expressed for this fraction in counts/min./mg. of fresh leaf tissue.

results it can be seen that the fluorine cannot be entirely removed from the Teflon surface, the remaining portion probably being fixed to the plastic molecule through isotopic exchange or through adsorption. The former hypothesis should not be excluded since the energy liberated by  $\text{F}^{18}$  during its disintegration is more than sufficient to break the C—C, C—H, or C—F bond, and, once broken, there can be a recombination with the  $\text{F}^{18}$  that is in the neighborhood of the ruptured bond.<sup>6</sup>

*Distribution of fluorine in cell constituents of tomato leaves.* Several experiments were conducted to determine the distribution of foliar-administered  $\text{F}^{18}$  and  $\text{F}^{19}$  among the cellular constituents of tomato leaves including cell walls, chloroplasts, mitochondria, microsomes, soluble proteins, and the supernatant. The experiments using  $\text{HF}^{19}$  gave information of distribution obtained after a long period of uptake (10 days), during which the plant accumulated 1,214 p.p.m. F, while the experiments in which  $\text{HF}^{18}$  was used indicated the sites of accumulation occurring during the first 3 hours of uptake. Table V shows these results, from which it can be seen, in both cases, that substantial quantities of  $\text{F}^{18}$  or  $\text{F}^{19}$  are ac-

<sup>6</sup> Personal communication from Dr. David Ballantine, Brookhaven National Laboratory.

cumulated in the cell walls, chloroplasts, mitochondria, and soluble proteins. The  $F^{18}$  in the supernatant of the nonwashed plants amounted to 80 per cent of the total. In the washed sample the  $F^{18}$  in the supernatant amounted to 20 per cent and the wash water to 68 per cent of the total fluorine.

Further studies were made on the chloroplast fraction by extracting the pigments with acetone in a Soxhlet extractor. The solvent was found to be practically free of  $F^{19}$ , suggesting that little or no fluorine is bound by the pigments or other acetone-soluble components in chloroplasts. Since the major constituent of chloroplasts is proteinaceous, a possible reaction between fluorine and proteins is suggested. Preliminary experiments using mild hydrolysis agents such as *N* HCl at room temperature on the chloroplast fraction have shown that a large part of the fluorine is readily removed. On the other hand, hydrolysis of the same fraction with 6*N* HCl at the boiling point for 18 hours followed by paper-partition chromatography for amino acids has shown the presence of the following compounds: cystine, lysine, histidine, arginine, aspartic acid, serine, glycine, hydroxyproline, glutamic acid, threonine, alanine, proline, tyrosine, methionine, valine, phenylalanine, leucine, and isoleucine. Work is now in progress using  $HF^{18}$  and centrifugal paper-partition chromatography to determine the possible existence of fluoroamino acids.

#### ACKNOWLEDGMENTS

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All of the analyses of  $F^{19}$  were done by Mr. Michael Grubiak, and the hydrolysis of chloroplast proteins and the subsequent chromatography of the amino acids were done under the supervision of Mr. Richard Mandl, to whom we wish to express our appreciation.

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# INCORPORATION OF TRITIUM-LABELED THYMIDINE INTO NUCLEI OF RUSTED BEAN LEAVES<sup>1</sup>

RICHARD C. STAPLES AND MYRON C. LEDBETTER<sup>2</sup>

Autoradiographic studies were made using tritium-labeled thymidine to determine the utilization of this metabolite by *Uromyces phaseoli* (Pers.) Wint. Although thymidine was not utilized by the fungus, it was incorporated in large quantities into the non-proliferating nuclei of the host. This report is made in light of the similar results recently obtained by Harding, Donn, and Srinivasan (2) showing the uptake of tritium-labeled thymidine by nuclei in injured lens epithelium.

Bean plants (*Phaseolus vulgaris* L. var. Pinto) were grown in composted garden soil in the greenhouse for 13 days at which time both primary leaves were lightly inoculated with uredospores of the bean rust fungus, *Uromyces phaseoli* (Pers.) Wint. Following the inoculation, the plants were placed overnight in a high humidity chamber at 16° C. and then returned to the greenhouse. Two days after the inoculation, one primary leaf was excised and a small polyethylene vial attached to the petiole stump with wax. One ml. of an aqueous solution of tritium-labeled thymidine containing 0.12 mc. H<sup>3</sup> was added to the vial. Specific activity of the thymidine was 172 millicuries per millimole. Fourteen days after the inoculation, pustules were excised from the leaf and prepared for microautoradiography as previously described (3).

Figure 1 A is a microautoradiograph of a rusted bean leaf showing a uredinium. Dark grains of silver demonstrate a low level of activity scattered throughout the leaf mesophyll, palisade, and epidermis. The large nuclei of the host show heavy incorporation of the label, though the fungus is clearly not labeled. Labeled nuclei were found in tissue beneath the uredinium, around the periphery, and even in elements of a vascular strand (Fig. 1 B), especially the phloem, some distance from the uredinium. The numbers of labeled nuclei gradually decreased in leaf areas distal to the uredinium.

As far as can be judged, these labeled host nuclei were not dividing and no reasonable explanation for the phenomenon can be offered except that it was due to injury of the host tissues by growth of the fungus. Nuclear labeling was stimulated by mechanical injury in the lens epithelium (2). The experiment could only be repeated when experimental conditions were rigidly adhered to, especially as to time of inoculation and administration of the thymidine. Thymidine concentration seemed to be without influence.

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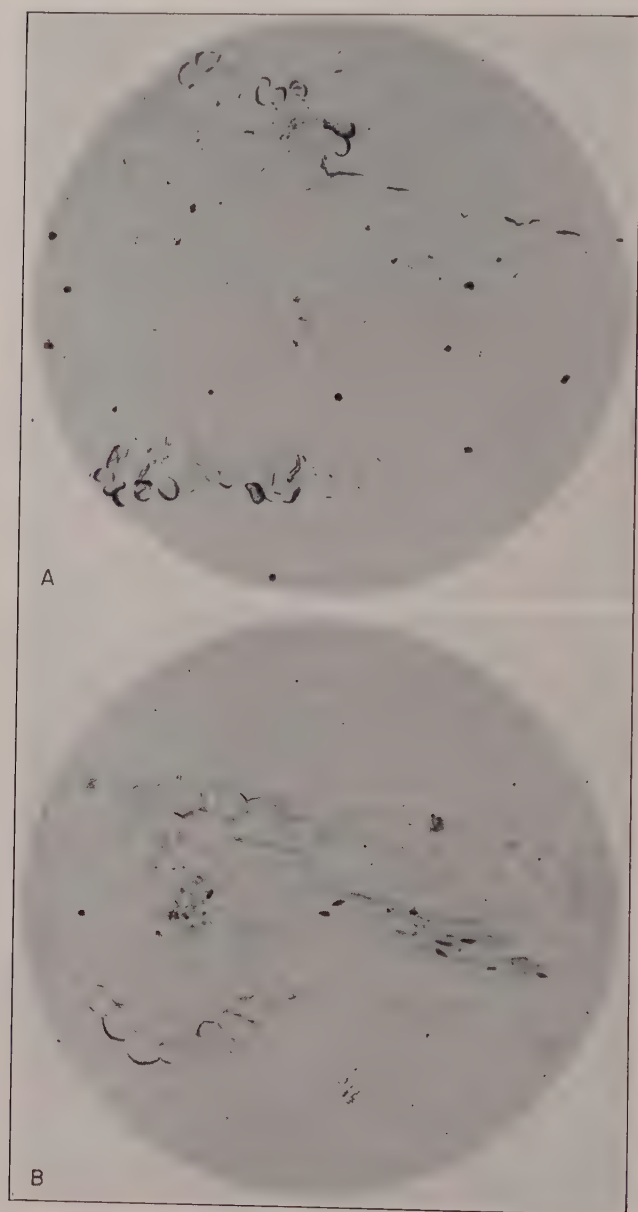


FIGURE 1. Microautoradiograph of cross section of bean leaf showing accumulation of label in nuclei. Dark areas indicate labeling ( $\times 145$ ). (A) Uredinium, showing accumulation of label in host nuclei and absence of label in fungus. (B) Vascular bundle showing labeled nuclei.

Thymidine was never observed to be taken up by the fungus whether on intact or excised leaves of various ages. This was interesting in view of the high accumulation of tritium from glycine by the fungus (3). Thymidine was never found in nuclei of the shoot apical meristem either when fed as a solution through the roots or through the petiole. Gifford (1) has reported a similar experience for *Chenopodium album*. The results suggest that deoxyribonuclear materials from the host nucleus do not penetrate the fungus or are not used in its metabolism.

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# THE PATTERN OF WATER CONDUCTION AND TRACHEIDAL ALIGNMENT IN DOUGLAS-FIR

W. H. HENDRICKSON<sup>1</sup> AND J. P. VITÉ<sup>2</sup>

## SUMMARY

The water conduction and tracheidal alignment of 15 seedlings, together with 36 trees ranging from 8 to 250 years, as well as the slope of grain of 367 weathered logs of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] were inspected by means of dye injection and mechanical techniques. The water was found to ascend along tracheidal conduits which are arranged in a distinct general pattern around the tree axis. However, a wide range of individual variation was observed.

A change in deviation of the alignment of the fibers with respect to the tree axis was found within the same growth ring from the tip of a tree down to its base, and within a transverse section from the pith towards the periphery according to the age of the wood section. Starting with a slight deviation to the right or a more or less parallel run at the tip and adjacent to the pith, the tracheidal conduits soon deviated to the left and later changed back to the right. Usually, a run parallel with the tree axis was evident in the wood aged between 45 and 90 years. The tracheidal conduits in the early springwood always deviated to the left of the later springwood and the summerwood.

In contrast to other conifers, the tracheidal conduits of adjacent growth layers were found to ascend in nearly the same sector along the entire stem, although the sector wound around the stem axis. Indications of an environmental influence upon the path of water conduction are discussed.

## INTRODUCTION

Continuing similar investigations on other conifers (10), specimens of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, ranging in age from 1 to 250 years were injected with 0.4 to 0.8 per cent Acid Fuchsin dissolved in ethyl alcohol and water. For specimens larger than 0.5 cm. in diameter, the procedure previously described (9) was used. Dye was supplied for several days through a hole drilled radially into the sapwood of the lower bole. Seedlings and small diameter shoots were injected using 0.4 mm. capillary tubes, or were split at the base and one-half of the stem was immersed in dye solution. Sometimes, in lieu of the injection procedure, the slope of grain was studied by means of a needle allowed to freely scribe a path along the fiber direction on the moist surface of freshly peeled stems (6).

The experiments were conducted in July and August of 1958 using Douglas-firs from the Boyce Thompson Institute's experimental forest at Grass Valley (elevation, 2100-2400 ft.), the Skillman Flat area of the Tahoe National Forest (elevation, 4400 ft.), and the Zeibright Mine

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TABLE I  
NUMBER, SIZE, AND AGE OF DOUGLAS-FIR TREES INVESTIGATED

| Size of trees   | No. of trees | Age in years | Basal diameter (inside bark), cm. | Height, cm. |
|-----------------|--------------|--------------|-----------------------------------|-------------|
| Seedlings       | 15           | 1-2          | 0.1-0.4                           | 8-14        |
| Saplings, small | 9            | 8-25         | 0.5-2.5                           | 30-167      |
| Saplings, large | 7            | 12-15        | 2.5-4.3                           | 257-387     |
| Poles           | 17           | 27-73        | 15-38                             | 900-3,000   |
| Timber trees    | 3            | 180-250      | 97-104                            | 3,600-4,700 |

tract of the New Verde Mines Company (elevation, 5000 ft.), all in Nevada County, California. The material investigated is presented in Table I.

Another study incidental to this report was an observation of the slope of grain as evidenced by the checks appearing in some weathered Douglas-fir logs located at the sawmill of the laboratory. One hundred and nineteen top logs, 100 butt logs, and 121 construction timbers sawn from Douglas-fir were selected for measurement. In addition, 23 larger butt logs were obtained at two local sawmills.

The general pattern of water conduction in Douglas-fir has been described (10). This investigation sought more detailed information regarding the path by which the sap ascends from the roots to the crown of a tree and sought to determine whether there were changes in this structure due to age and environmental influences. As shown earlier, the dye solution injected into a conifer ascends via the tracheids, or "tracheidal conduits," the run of which is identical to the run of the tracheids (8). When aligned other than parallel to the tree axis, the fibers necessarily have a deviation or slope. This is commonly known as slope of grain or spiral grain. The distribution of the injected dye in a tree shows the pathways by which the moisture ascends as well as the pattern in which the tracheids are arranged in relation to the tree axis. Since Douglas-fir is one of the more important forest trees, several investigations have been made on its wood structure. Among the recent publications, those on wood structure and properties in relation to age and environmental influences have been of special interest (1, 4, 5, 7). For general information, two regional monographs were consulted (2, 3).

#### THE PATTERN OF TRACHEIDAL ALIGNMENT

In seedlings and in the young growth at the top of a tree the tracheidal conduits were more or less parallel to the tree axis or deviated at an angle of 1 to 3 degrees to the right. When a right deviation was shown, it may have existed for the first 10 years of growth. Accordingly, in certain small trees, the entire length of the stem showed this right deviation. Further attempts to study the alignment of tracheids in the leader of older trees and

in seedlings were not conclusive enough to substantiate the finding of Northcott (7) that a left tracheidal alignment also may occur in the wood of 1- and 2-year-old Douglas-fir seedlings.

Furthermore, below the tip of a tree, the tracheidal alignment changed within a given growth ring to the left of the tree axis. Usually, the left deviation increased for a short distance but decreased again in the next lower section until the fibers turned into a more or less parallel position to the tree axis somewhere in the middle portion of a tree where the wood age averaged between 40 and 90 years. Then, deviation to the right increased with the age of the wood towards the base of the tree.

The changes in direction and deviation of the tracheidal alignment found in a vertical aspect within the same growth ring were also observed radially across all growth rings from the pith towards the periphery of the trunk (Fig. 1 A). In growth rings near the center, the tracheids deviated to the right or ran nearly parallel to the stem axis (Fig. 2 A), turned in following growth rings to the left (Fig. 2 A and B), and changed to the right again after passing a position parallel to the tree axis (Fig. 2 C). Alignment to the right was typical for the outer growth rings in the lower trunk of an older tree (Fig. 2 D).

All trees investigated showed the same general pattern. The tracheidal alignment in a given portion of a trunk depended on the number of previous growth rings or the wood age. A change in the tracheid direction from the right to the left side of the tree axis in the youngest xylem layer soon changed from the left to the right. Thus, the tracheid direction was more or less predictable for wood of various ages. However, the degree in which the tracheidal conduits deviated varied widely in individual trees. A measurement of the slope of grain on the surface of 363 Douglas-fir logs and timbers of different ages showed the range of this variability for local specimens (Table II). An average deviation of  $4.00^\circ$  to the left (with a range of  $2^\circ$  to the right and  $8^\circ$  to the left) was developed in trees 11 to 20 years old; a position parallel to the tree axis was manifested at about 70 to 80 years, while trees 161 to 320 years of age showed an alignment of  $4.78^\circ$  to the right (with a range of  $3^\circ$  to the left and  $14^\circ$  to the right). Such differences were anticipated on the basis of Northcott's observations of the slope of grain in Douglas-firs of the same locality (7).

Under extreme circumstances, an older tree may have only left-oriented or only right-oriented tracheids although showing the same general pattern of tracheidal alignment otherwise. Thus, a 73-year-old Douglas-fir of very slow growth, 17 cm. diameter at breast height (d.b.h.) and 19 meters tall, did not exhibit any left deviation in the sapwood; near the center, the fiber direction started out with a slight deviation to the right and increased to stronger deviation to the right towards the periphery of the sapwood. On the other hand, a 180-year-old tree of continuously

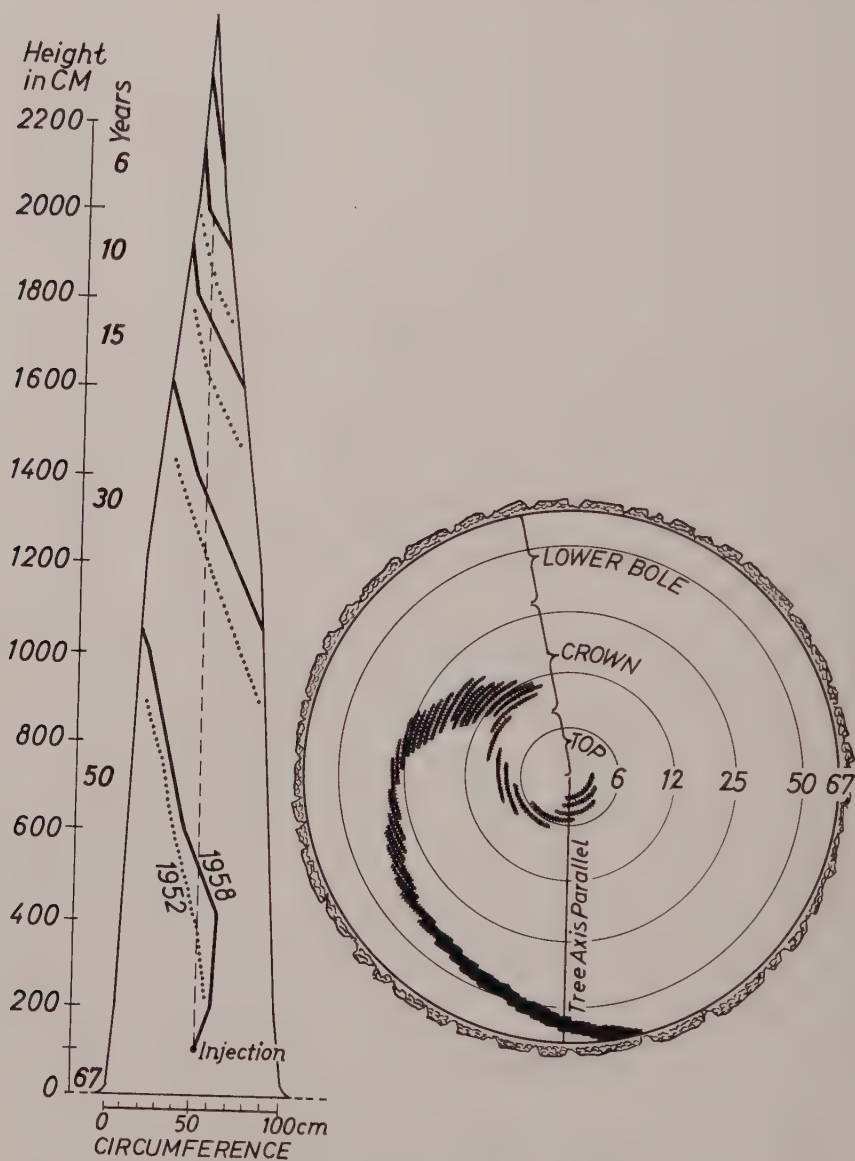


FIGURE 1. Run of the tracheidal conduits in the growth rings of the years 1952 and 1958 of a 67-year-old Douglas-fir as obtained by dye injection (left), and scheme of fiber orientation of the same trunk as found in the transverse section (right). Vertical numbers in drawing on the left and horizontal numbers in drawing on the right indicate age of wood in years.

TABLE II  
AVERAGE AND INDIVIDUAL SLOPE OF GRAIN IN DEGREES AND DIRECTION OF 363 DOUGLAS-FIR LOGS  
AND TIMBERS OF DIFFERENT AGE AS MEASURED FROM SURFACE CHECKS

| Age of wood<br>in years | Average slope<br>of grain in<br>degrees and<br>direction | Number of trees with the same slope of grain in degrees and direction |   |    |    |    |    |    |    |    |    |              |    |    |    |    |   |   |   |   |    | Total |    |     |    |
|-------------------------|----------------------------------------------------------|-----------------------------------------------------------------------|---|----|----|----|----|----|----|----|----|--------------|----|----|----|----|---|---|---|---|----|-------|----|-----|----|
|                         |                                                          | To the left                                                           |   |    |    |    |    |    |    |    |    | To the right |    |    |    |    |   |   |   |   |    |       |    |     |    |
|                         |                                                          | 9                                                                     | 8 | 7  | 6  | 5  | 4  | 3  | 2  | 1  | 0  | 1            | 2  | 3  | 4  | 5  | 6 | 7 | 8 | 9 | 10 |       | 11 | 14  |    |
| 5-10                    | 3-14 left                                                |                                                                       |   |    |    |    | 2  | 1  | 2  |    | 2  |              |    |    |    |    |   |   |   |   |    |       |    | 7   |    |
| 11-20                   | 4-00 left                                                |                                                                       |   |    |    |    | 3  | 1  | 3  | 9  | 6  | 12           | 4  | 2  |    | 1  |   |   |   |   |    |       |    |     | 42 |
| 21-40                   | 2-90 left                                                | 1                                                                     | 2 | 6  | 5  | 18 | 9  | 18 | 11 | 14 | 11 | 14           | 11 |    | 2  | 1  | 2 | 1 |   |   |    |       |    | 101 |    |
| 41-80                   | 1-04 left                                                |                                                                       | 1 | 10 | 4  | 17 | 6  | 15 | 16 | 11 | 35 |              |    | 7  | 6  | 9  | 9 | 5 | 3 |   | 1  |       |    | 155 |    |
| 81-160                  | 0-73 right                                               |                                                                       | 1 |    | 1  | 2  |    |    | 1  | 4  | 6  |              |    | 6  | 6  | 6  | 1 | 2 | 3 |   |    |       |    | 44  |    |
| 161-320                 | 4-78 right                                               |                                                                       |   |    |    |    |    |    | 1  |    | 2  |              |    | 1  | 2  | 1  | 3 | 1 | 1 | 1 | 1  |       |    | 14  |    |
| Total                   |                                                          | 2                                                                     | 6 | 17 | 13 | 48 | 22 | 49 | 35 | 35 | 54 |              | 15 | 14 | 18 | 10 | 8 | 5 | 8 | 1 | 1  | 1     |    | 363 |    |



rapid growth, 100 cm. d.b.h. and 36 meters tall showed only left deviation.

Within a growth ring, the tracheids of the early springwood were found to deviate always to the left of the later springwood and the summerwood (Fig. 2 A). The angle of this deviation seemed to be greater in the younger parts of the trunk in the crown region but was small even in extreme cases, ranging from  $0.5^{\circ}$  to  $0.6^{\circ}$ .

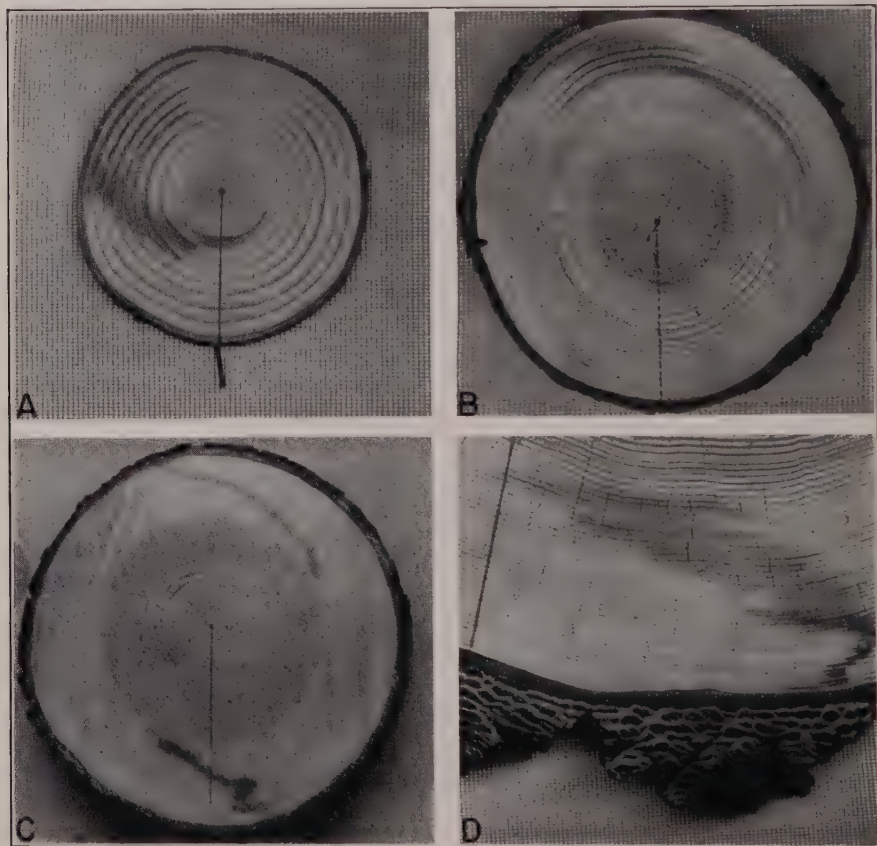


FIGURE 2. Transverse sections 4 meters above radial dye injection into the lower bole of a young tree, wood-age 12 years (A), a sapling, wood-age 25 years (B), a pole-size tree, wood-age 56 years (C), and an old-growth Douglas-fir, wood-age 176 years (D). The pencil mark running from the pith toward a nail in the bark indicates the situation of the injection parallel to the tree axis.

#### THE PATTERN OF WATER CONDUCTION

A preliminary study had shown that the pattern of water conduction in the genera *Tsuga* and *Pseudotsuga* differs from that of other conifers. The main difference was found in the pattern in which the tracheidal conduits are arranged in subsequent growth layers. Usually, the angle of tracheid

deviation in adjacent growth rings decreases or increases only to the extent that radially adjacent conduits wind around the tree axis in the same sector (Fig. 2). Therefore, this type of water conduction was designated as "winding, sectorial ascent" (10).

Further studies in Douglas-fir showed that this pattern holds generally true for the trunk in the region of the active crown but there was a larger variability in the lower bole and at the tip of a tree. In the tip region, the tracheidal conduits of subsequent growth rings were usually arranged in a semispiral, turning from the periphery counterclockwise towards the pith (Figs. 1 B and 2 A). In the crown region below the top, a more or less parallel run of the tracheidal conduits prevailed (Fig. 2 B). The deviation of the tracheidal conduits of the early springwood to the left of the later springwood and the summerwood became most obvious. In the lower bole, the tracheidal conduits ascended in the same sector (Fig. 3 A), or their deviation changed continuously to a semispiral running clockwise from the periphery of the sapwood towards the heartwood (Fig. 3 E). Also, reverse changes occurred (Fig. 3 C). Naturally, the tracheidal alignment in the lower bole determined the further distribution of the water transported from a given sector of the tree base. Where, in the lower bole, the tracheidal conduits of the various growth layers kept within a narrow sector, dye was distributed to a limited section of the crown region only (Fig. 3 A and B). A semispiral arrangement of the conduits in the lower bole of a tree distributed water over a large section, in extreme cases over the whole cross section of the trunk in the crown region (Fig. 3 E and F). This closely resembled the distribution found in other Pinaceae (9, 10). Semispiral arrangement with reverse changes of tracheidal alignment in the lower bole resulted in an intermediate pattern of water distribution in the crown region (Fig. 3 D).

Attempts to correlate the distinct variations mentioned above to environmental influences failed, probably because the rate of diameter growth was used as the only indicator of differences in environment. However, some interesting indications were obtained by carrying out a limited investigation on the differences in the pattern of injected dyes in trees suppressed by dense overstory, in trees previously released from overstory, and in pruned trees. Two small suppressed trees, two larger ones, two released trees, and a top-pruned tree were dye-injected. The pattern of the suppressed trees fitted within the range established for the species. The released and top-pruned trees yielded dye patterns which were different, and from these, tentative conclusions are offered.

Release for the two trees was obtained by logging mature overstory trees 8 years previously. An 84-year-old Douglas-fir, 21 cm. d.b.h., was released from an overstory of two larger trees located within 3 meters. The stump diameter of the overstory trees was approximately 1 meter. The release did not produce an increase in the width of the growth rings.

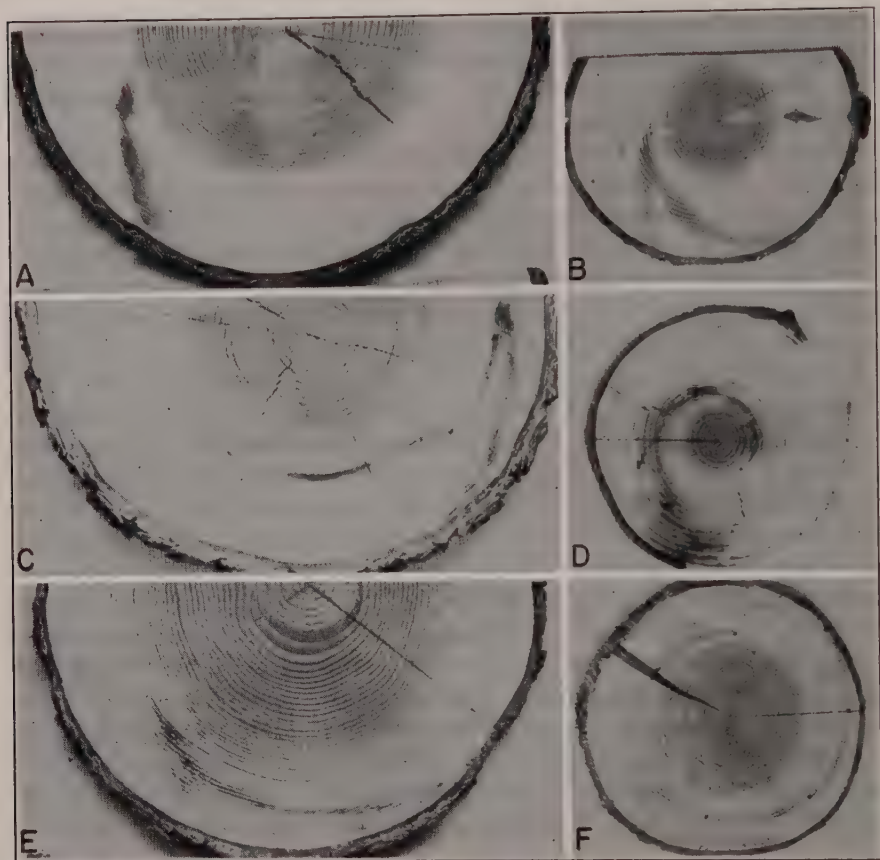


FIGURE 3. Transverse sections 4 meters (A, C, E) and 12 meters (B, D, F) above dye injection into the lower bole of about 60-year-old Douglas-firs of different wood structure. A, B: Tree in which the tracheidal conduits of the sapwood are confined to a narrow sector. C, D: Tree with reversing semispiral arrangement of the tracheidal conduits in the lower bole (C) resulting in partly narrow, partly semispiral distribution of water in the crown region (D). E, F: Tree with extreme wide distribution of the tracheidal conduits in the lower bole (E) and the crown (F).

However, the alignment of the tracheidal conduits was modified. Having a right deviation of fiber at the time of release, the tracheidal conduits thereafter increased the speed with which they moved right.

A 50-year-old tree, 16 cm. d.b.h. and released from a tree of 1 meter stump diameter, produced somewhat wider growth rings. At the time of release it had not yet developed right-deviating conduits in the lower bole. After release, however, the reduction in left deviation in each successive growth ring (moving outward) proceeded more rapidly resulting in an increase in the rate at which the tracheidal conduits moved to a right orientation.



The top-pruned tree apparently lost its top in the course of logging 8 years ago. The d.b.h. of this tree was 33 cm., the age 70 years. During the years prior to the top damage, the orientation of the tracheidal conduits had moved successively right and was in the year of damage nearly parallel to the tree axis. Following the general pattern, it would now be expected that a right deviation would be developed and this deviation in successive years would be stronger. Instead, pruning apparently upset the alignment shift so that the grain remained straight for the 8 successive years.

Similarly, Douglas-fir grown in the open pine forests of the lower elevations exhibited a more parallel run of the tracheidal conduits of subsequent growth layers (less tendency to change) while trees of the denser pine-fir stands tended to show a semispiral arrangement. The former usually had deep crowns, the latter short crowns resulting from competition for light.

Altogether, these observations give an indication that environmental factors such as light influence the size and shape of the crown. This in turn influences the pattern of tracheidal alignment and water conduction of a single tree as well as the trees of a certain locality. Therefore, the valuable observation of Northcott (7, p. 350) that "trees of the same species, grown in widely separated regions, appear to develop significantly different severities of spiral grain" should not be considered from a genetical point of view only.

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# THE SULFUR METABOLISM OF INSECTS. V. THE ABILITY OF INSECTS TO USE SULFATE IN THE SYNTHESIS OF METHIONINE<sup>1</sup>

THOMAS H. HAINES, S. MARK HENRY, AND RICHARD J. BLOCK<sup>2</sup>

## SUMMARY

$\text{Na}_2\text{S}^{35}\text{O}_4$  was administered to 13 species of insects. The Japanese beetle was able to utilize the sulfur of  $\text{Na}_2\text{S}^{35}\text{O}_4$  for the synthesis of methionine and other divalent sulfur-containing compounds.

Of the remaining insects, only the cockroaches, known to contain intracellular symbionts, were able to effect this reduction.

## INTRODUCTION

Although various organic sulfur-containing compounds have been found in animals after the administration of  $\text{Na}_2\text{S}^{35}\text{O}_4$ ,  $\text{S}^{35}$ -methionine has not been reported in the absence of bacteria (1). Unless methionine can be made from sulfate-sulfur by the animal *per se*, administration of sulfate merely has a sparing action on the methionine requirements. It has been previously shown (1) that *Blattella germanica* (L.) is not able to synthesize methionine in the absence of its intracellular symbionts. In this investigation, five additional species of cockroaches and eight other insects were tested for their ability to synthesize methionine from  $\text{Na}_2\text{S}^{35}\text{O}_4$ .

## EXPERIMENTAL

*Insects.* The following insects were used:

- Orthoptera: Florida cockroach, *Eurycotis floridana* (Walker)  
Oriental cockroach, *Blatta orientalis* L.  
Australian cockroach, *Periplaneta australasiae* (F.)  
Madeira roach, *Leucophaea maderae* (F.)  
Beetle roach, *Diploptera punctata* (Eschscholtz)
- Lepidoptera: Banded woollybear, *Isia isabella* (J. E. Smith)  
Southern armyworm, *Prodenia eridania* (Cram.)
- Coleoptera: Mexican bean beetle, *Epilachna varivestis* Muls.  
Broad-necked root borer, *Prionus laticollis* (Drury)  
Ground beetle, *Carabus* sp.  
Japanese beetle, *Popillia japonica* New.  
Yellow mealworm, *Tenebrio molitor* L.

<sup>1</sup> The data presented here represent a part of a dissertation submitted by S. Mark Henry to the Faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup> Aided by grants E-2608 and RG-4987, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

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Hemiptera: Large milkweed bug, *Oncopeltus fasciatus* (Dall.)

The beetles, *Popillia*, *Carabus*, *Tenebrio*, and *Prionus* were in the adult stage. All other insects used in the investigation were nymphs or larvae.

*Methods.* Five to ten  $\mu\text{l}$ . (1  $\mu\text{c.}/\mu\text{l.}$ ) of carrier-free  $\text{Na}_2\text{S}^{35}\text{O}_4$  were injected into the hemocele of the cockroaches, beetles, and woollybear caterpillar with a micrometer controlled syringe. The southern armyworm and Mexican bean beetle larvae were maintained on filter paper wet with a  $\text{Na}_2\text{S}^{35}\text{O}_4$  solution. After 24 hours the insects were homogenized in hot 80 per cent (v/v) ethanol containing 0.001 per cent (w/v) *N*-ethylmaleimide and the insoluble material was removed by filtration (1). The residue was then washed and hydrolyzed with 3*N* HCl.

The radioactive metabolites were identified by means of autoradiograms of two-dimensional chromatograms (1).

### RESULTS

*Cockroaches.* The five species of cockroaches produced methionine, cystine, and the other sulfur-containing compounds when given  $\text{Na}_2\text{S}^{35}\text{O}_4$ , as was previously reported for *Blattella* and *Periplaneta* (1).

*Japanese beetle.* This insect, in which intracellular symbionts have not been reported, formed methionine, cystine, and traces of glutathione, sulfite (or thiosulfate), and taurine from  $\text{Na}_2\text{S}^{35}\text{O}_4$ .

*Other insects.* None of the other insects tested converted the sulfur of  $\text{Na}_2\text{S}^{35}\text{O}_4$  into methionine or even cystine.

### DISCUSSION

It appears from the present report and previous studies (1) that the characteristic intracellular symbionts of cockroaches enable this family of insects to incorporate sulfate-sulfur into methionine and other organic compounds. On the other hand, the remaining insects tested were unable to effect this conversion except for the Japanese beetle. Although the authors are not aware of any reports in the literature that Japanese beetles contain intracellular symbionts, this possibility cannot be excluded at the present time. Furthermore, the fact that the  $\text{Na}_2\text{S}^{35}\text{O}_4$  was administered parenterally does not exclude absorption via the malpighian tubules into the gut and subsequent reabsorption of methionine synthesized by the gut microbiota. Experiments are in progress to characterize the metabolism of sulfate in the Japanese beetle.

The authors wish to thank Dr. Albert Hartzell for identification of the insects.

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# BIOASSAY AND BIOCHEMICAL STUDIES OF EXTRACTS OF PEACH SEEDS IN VARIOUS STAGES OF DORMANCY

FLORENCE FLEMION AND DOROTHY STROUT DE SILVA

## SUMMARY

The various components in methanolic extracts of seeds of peach [*Prunus persica* (L.) Stokes] which separated on paper chromatograms were eluted and bioassayed. Irrespective of whether the seeds were dormant, partially dormant, non-dormant, or in secondary dormancy, eluates from near the base line at Rf values of 0.18 and 0.34 stimulated the elongation of wheat coleoptile sections while those from a higher region—Rf 0.36 to 0.58—inhibited development. The stimulatory materials were eluted from an area on the chromatogram where the sugars and some of the amino and organic acids had traveled. Organic acids were also present in the zone of inhibition.

When excised peach embryos with or without cotyledons were assayed, substances toxic at relatively high concentrations but ineffectual at lower concentrations were eluted from two regions on the chromatograms which had Rf values of 0.10 and between 0.26 and 0.60. No response was obtained when the various eluates were applied directly to the tips of actively-growing peach seedlings. In these investigations there was no indication of any direct relationship between the phenomenon of dormancy and the materials extracted from the seeds which had deleterious or inhibiting effects in the bioassays.

Seeds as well as seedlings of peach were treated with various solutions of naringenin, a substance recently isolated from dormant flower buds of peach (14), and of the glucoside, naringin. There was no indication that the concentrations used under the conditions described had any effect on either germination or subsequent development.

In quantitative studies, the various amino acids, organic acids, and phosphates present in dormant peach seeds gradually increased in amounts as the period of chilling at 5° C. progressed. The mobilization of the reserve materials also occurred under less favorable conditions which produced dwarfed or semi-dwarfed seedlings instead of the normal plants obtained from adequately chilled seeds. During the germination of chilled and unchilled seeds, which produced normal and dwarfed plants respectively, there were tremendous increases in amounts of many of these materials while with others the levels differed considerably. It is not known whether the differences are significant in causing a breaking of the rest period or whether the various amounts reflect a secondary rather than a primary role of these substances in overcoming dormancy.

All of the investigations were conducted with a variety of peach seed which has an intermediate chilling requirement.

## INTRODUCTION

The beneficial effect of low temperature in overcoming the dormancy of seeds and buds of different north temperate zone plants has long been known, and many investigations have been concerned with the various changes associated in the breakdown of the reserve materials, the accumulation of the soluble materials during the chilling period, and the

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possible role of growth promoters and inhibitors. Soluble substances have been extracted from seeds and buds of peach which inhibited or promoted the development of sections of the pea epicotyl (13) and the coleoptile of oats (3, 4, 17) and wheat (14, 29, 31). Various deductions have been made concerning the relationship of these materials with the breaking of the rest period.

The present study was undertaken with peach [*Prunus persica* (L.) Stokes] seeds at different stages of dormancy as well as those in which secondary dormancy had been induced. The constituents in the extracts which separated on the paper chromatograms were assayed for effects on the development of not only the coleoptile sections of wheat but also the embryos and seedlings of peach. Since the sprayed chromatograms indicated the presence of amino acids, organic acids, and sugars in the areas from which some eluates were inhibitory while others were stimulatory, these compounds were identified qualitatively, and, in the case of the acids, quantitative determinations were obtained. The data are presented below together with illustrations of the different types of plants obtainable from the seeds used in these investigations.

#### MATERIALS AND METHODS

Peach seeds of a propagated selected wild strain grown in Tennessee and known as "Superior Peach Pits" were purchased and stored dry in closed containers at 5° C. (41° F.). Upon removal of the hard outer coat (pericarp) the seeds with inner coats intact were mixed in a moist granulated peat moss and chilled at 5° C. for various periods ranging from 0 to 12 weeks. Secondary dormancy was induced when, after partial chilling of 4 to 6 weeks at 5° C., the seeds were transferred and maintained in a moist condition at 25° C. (79° F.). For the production of seedlings in the greenhouse the seeds pretreated in these various ways were then excised and germinated on moist filter paper in Petri dishes at room temperature prior to planting in the soil in the greenhouse (10).

*Extraction and concentration procedure.* The seeds or buds were frozen rapidly by immersion in beakers held in a mixture of dry ice and methanol and then immediately ground in a VirTis 45 homogenizer with cold 80 per cent methanol or ethanol. After the homogenates (12 ml. per gram of fresh tissue) had been held for at least 72 hours at -10° C. the supernatant was filtered. Whereas the methanolic extracts were immediately reduced to a 10-ml. volume, by using a rotating vacuum tube evaporator, then held at 8° C. until bioassayed, the ethanolic extracts were passed through ion-exchange resins for the separation of the amino (21, 22) and organic (19, 25) acids. Upon passage through a 1 × 20-cm. cation-exchange column of Dowex 50-X4, 20 to 40 mesh, hydrogen form, at a rate of flow approximately 1 ml. per minute, the amino acids were retained and then eluted

and taken to dryness with a stream of air. Upon resuspension in 0.5 or 1 ml. of distilled water they were held in the freezing compartment of the refrigerator.

Following passage through the cation-exchange resin, each ethanolic extract was passed slowly (1 ml. per minute) through an anion-exchange column (19, 25) of Dowex 1-X2, 50 to 100 mesh, formate form (bedded in ethanol). The organic acids were then rapidly eluted from the resin (19), taken to dryness, resuspended in 30 ml. of distilled water, and, after passing through a resin column of Dowex 1-X10, 200 to 400 mesh, formate form (bedded in water), they were removed by gradient elution (19). Following evaporation of the eluting agent from the 2-ml. fractions, each residue was resuspended in 1 ml. of distilled water and titrated with 0.01*N* NaOH. All of the acid from a given peak was pooled, taken to dryness, resuspended in either 0.5 or 1 ml. of distilled water, and then stored at  $-2^{\circ}$  C. In order to remove the sodium ions (25), Dowex 50-X4, 20 to 50 mesh, hydrogen form, was added.

Dry weight determinations were obtained from duplicate lots of 100 intact seeds each taken at the time of homogenization which were then dried at  $100^{\circ}$  C. until constant weight was obtained.

*Bioassays.* Twenty-microliter aliquots of the concentrated extracts were spotted on Whatman No. 1 chromatography paper as described by Vlitos and Meudt (28), and the papers were then irrigated for 16 hours in either isoprophyl alcohol—ammonia—water (80:5:15 v/v) or *n*-butanol—acetic acid—water (60:25:15 v/v). The chromatograms were examined for fluorescence by using two mercury vapor lamps each provided with ultraviolet filters. The wavelengths were approximately 220 to 300 and 280 to 400 millimicrons. There was a fluorescent spot, visible under the long wave ultraviolet light, on all of the chromatograms, and the papers having similar *R<sub>f</sub>* values (see Fig. 1) for this unknown material were cut into longitudinal strips 2 cm. wide, then crosswise into 2-cm. pieces—from 2 cm. below the origin to 24 cm. above. A piece the same size cut from the paper where only the solvent had ascended served as control. One or more of the 2×2-cm. pieces from a given region from different papers were placed in one tube which contained 2 ml. of glass distilled water and after elution, for at least 24 hours at  $10^{\circ}$  C., the papers were removed and the eluate tested for biological activity. Both wheat coleoptiles and peach embryos were used in the bioassays.

In the case of peach, the embryos of both chilled and unchilled seeds were excised and either both cotyledons were removed (ecotyledonized) or one-half of one cotyledon was retained. These were placed in the various eluates and rotated on a roller tube (12 revolutions per hour) either in the dark at  $20^{\circ}$  C. or in the light at room temperature. It was not advisable to use intact peach embryos since because of size only one could have been

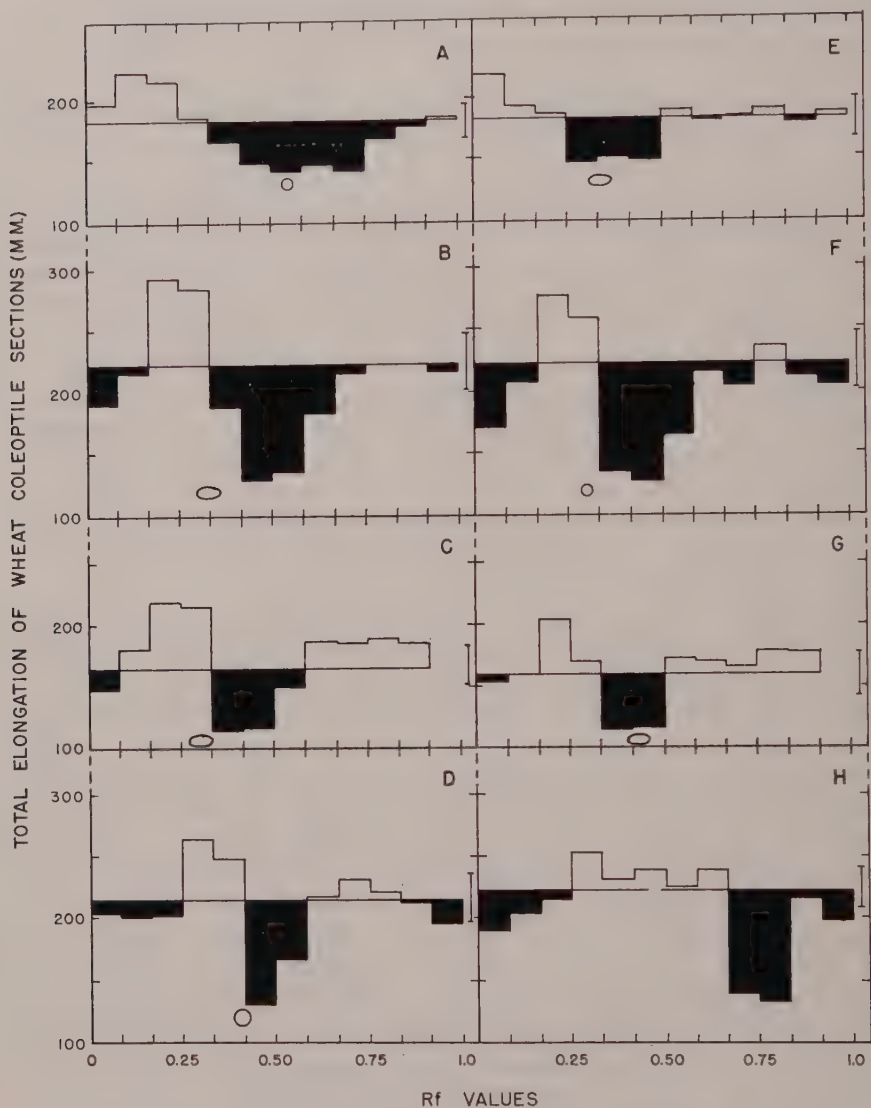


FIGURE 1. Wheat coleoptile assay of eluates from chromatograms of methanolic extracts of seeds (A to G) and dormant apical buds (H) of peach. Seed pretreated ○ (A, B), 14 (D), 53 (C), and 63 (E, F) days at 5° C. and (G) 45 days at 5° C. +8 days at 25° C. Butanol (A, E) and isopropanol (B, C, D, F, G, H) solvents. Equivalent amounts of dry weight: 290 (A, B), 280 (E, F), 190 (G), 188 (D), 150 (C), and 63 (H) mg. per ml. of eluate. Horizontal line: total elongation of growth of controls. Vertical base to right of histogram indicates units (mm.) required for significant growth changes at 5 per cent critical difference.

placed in each tube and besides, it has been shown that removal of 75 per cent of the cotyledonary tissue does not impair germination.

When wheat coleoptile sections were used, they were obtained by soaking seeds [Hard Red (Selkirk) wheat, *Triticum aestivum* (L.), 1957 North Dakota crop] for approximately six hours at room temperature in tap water, after which they were held on damp filter paper in moist chambers (wrapped with photographic light-proof cloth) at 25° C. in a temperature-controlled oven in the dark room. After 60 hours when the coleoptiles were approximately 25 to 30 mm. long, sections were excised (2, p. 417) in a room similar to that used by others (18, 28). The light source consisted of two 40-watt Westinghouse green fluorescent tubes each wrapped individually with two layers of amber and two layers of green cellulose plastic sheets of window shading and in order to reduce the light intensity the entire lamp was covered with four layers of cheesecloth. The 4-mm. section at the tip of the coleoptile was discarded while the next 6-mm. piece was placed in glass distilled water, and, when a sufficient number had been collected for a given experiment, they were transferred at random (usually 20) to each of the various eluates. Tubes containing coleoptile sections and eluates were rotated on the roller tube in a dark room (20° to 25° C.) for 20 hours, and then with the aid of a photographic enlarger the sections were measured. Since the solvent on the chromatography paper ascended approximately 24 cm. from the spotted line, each of the 12 spacings indicated along the base lines of the histograms in Figure 1 represents a distance of 2 cm. and has an Rf value of 0.083.

The statistical method which utilizes the range as a measure of standard deviation was used for the calculation of significant inhibition or stimulation of the various eluates. The sum of the ranges was multiplied by the appropriate entry shown by Tukey (27, p. 91, Table 2), and the factor thus obtained was the 5 per cent critical difference between any two totals. The difference was subtracted from as well as added to the elongation of the controls; however, this method also permits comparison of any two treatments with each other regardless of the control. With a less rapid but more efficient statistical technique it might have been possible to demonstrate significant differences with smaller amounts of extracts than those indicated in the text below.

*Amino acids.* Aliquots of the resuspended residue were spotted on Whatman No. 3 paper for two-dimensional descending chromatography. The techniques employed for both qualitative and quantitative analyses were those described by Porter, Margolis, and Sharp (22).

*Organic acids and phosphates.* The tentative identification of the organic acids which had been made by their order of displacement during the gradient elution process was verified by ascending partition chromatography on Whatman No. 1 paper (25). For the detection of malic and citric



acids and phosphates, the papers were developed in Stark, Goodban, and Owens' solvent F (26) for 16 hours, whereas, four to five hours in their solvent A was used for 2-pyrrolidone-5-carboxylic acid (PCA) (pyroglutamic). The location of these various acids was determined by spraying the papers with bromphenol-blue (19). For the orthophosphates a positive test was obtained when a yellow elongated spot obtained with ammonium molybdate turned blue when exposed to hydrogen sulfide fumes. No further identification or separation of the phosphates was undertaken.

*Sugars.* The filtrate from which both the amino and organic acids had been removed was concentrated by a stream of air at room temperature to 10 ml. and then stored at 8° C. For one-dimensional ascending sugar chromatograms, small sheets (27 × 40 cm.) of Schleicher and Shuell No. 598 paper were irrigated three times (drying after each exposure) in butanol-pyridine-water (32, p. 35), and for descending chromatography full size sheets of the same paper were run in large tanks (22, p. 466) in *n*-butanol—acetic acid—water (40:10:50) for 30 hours. Color development occurred within 20 minutes at 90° C. when the thoroughly dried papers were sprayed with naphthoresorcinol (10 ml. of 0.2 per cent in absolute EtOH + 1 ml. 85 per cent phosphoric acid). With this indicator the ketoses (fructose and sucrose) appeared brick red while the aldoses (such as glucose, galactose, etc.) were turquoise blue. Standard solution of various sugars was spotted alongside as well as on the spots of the unknowns. No quantitative studies were undertaken.

*Chemical treatments.* Naringenin was obtained from Delta Chemical Works, New York, N. Y., and naringin from Eastman Organic Chemicals, Rochester, N. Y. When the materials were applied as a spray under pressure, all parts of the peach seedling (stem, apex, upper and lower surfaces of the leaves) were thoroughly wetted, then allowed to dry before further application. Thus, in order to apply 50 ml. of solution to 24 seedlings on a given day, at least an hour or longer was required. For vascular feeding, several strands from a glass wick were twisted together and one end of this very thin wick was inserted, with the aid of a needle, through and beyond the stem of an actively-growing peach seedling while the other end was held in the solution which was in a glass vial resting on top of the soil.

## RESULTS

When the methanolic extracts were chromatographed in the basic isopropanol-ammonia solvent, there were two regions on the chromatograms which when eluted had distinctly different effects on the development of wheat coleoptile sections. This occurred irrespective of whether the extract was obtained from dormant or chilled seeds. Eluates from a zone with an approximate *R<sub>f</sub>* value from 0.19 to 0.34 stimulated elonga-

tion, whereas those from a higher region—Rf 0.36 to 0.58—inhibited development (Fig. 1 B, C, D, F, and G). Similar effects were also obtained when an acidic (butanol-acetic) solvent was used; however, lower Rf values (0.00 to 0.26) were obtained for the region of stimulation. When chromatographed aliquots of the extracts contained approximately equivalent amounts of milligrams of dry weight (calculated on the basis of dry weight of the tissue prior to extraction), the responses were more pronounced with eluates from the paper irrigated with the basic (Fig. 1 B and F) than with the acidic (Fig. 1 A and E) solvent. Equivalents of 280 and 290 mg. of dry weight in each milliliter of eluate not only inhibited elongation (Fig. 1 B and F) but were also frequently toxic; that is, discoloration of the tissue occurred. Smaller amounts of 150 to 190 mg. were also inhibitory (Fig. 1 C, D, and G); however, the tissue remained healthy for the duration of the test. Very slight inhibition sometimes occurred with 93 mg. while 46 mg. were ineffectual.

When peach was used instead of the wheat coleoptile sections, the embryos of both chilled and unchilled seeds were placed in the various eluates obtained from chromatograms run in the basic solvent. The tubes were rotated for four days when part of the cotyledons had been retained while longer periods up to two weeks were maintained when the embryos had been ecotyledonized. When each milliliter of eluate contained equivalent amounts of dry weight ranging from 170 to 340 mg., there was a region on the paper—Rf value from 0.26 to 0.60—which was apparently toxic as indicated by discoloration of the embryos. Deleterious effects were not obtained with equivalent amounts of 60 to 125 mg. of dry weight. The embryos (each with only part of one cotyledon) which survived the various soaking treatments were placed on moist filter paper in Petri dishes at room temperature and then, following adequate root development, they were transferred to soil in the greenhouse. Subsequently, there was no indication whatsoever in any of the 700 plants obtained in this manner that seedling development had been accelerated, retarded, or hindered. When ecotyledonized embryos remained in the eluates for 6 to 15 days, the substances from two regions on the chromatograms—Rf 0.10 and 0.30 to 0.60—produced discoloration and subsequent deterioration. This occurred with equivalent amounts of 60 to 120 mg. dry weight per ml. of eluate; however, with 45 mg. there was no indication of toxicity.

Aliquots of extracts from seeds chilled for 54 days at 5° C. were placed on paper chromatograms so that subsequently each milliliter of eluate contained an equivalent amount of 342 mg. of dry weight. To the actively-growing tips of peach seedlings from unchilled seeds the eluates were applied as drops one at a time several times daily until a total of 20 drops from each eluate had been applied to each of eight seedlings. The 112 treated plants were held for subsequent observation, but there was no

evidence of any inhibitory or stimulatory effects since they were all similar to the water-treated and untreated controls.

Methanolic extracts of dormant apical buds of normal peach seedlings also produced zones on the chromatograms which, when eluted, either stimulated or inhibited the development of wheat coleoptile sections (Fig. 1 H). The region from which inhibition was obtained had a higher Rf value (0.68 to 0.85) than that obtained with the seed extracts (Fig. 1 A to G) and, in addition, the response was obtained with smaller amounts, that is, with the equivalent of 63 mg. dry weight per ml. of eluate. There was no effect on subsequent seedling development when peach embryos (with one-half of one cotyledon) were tested in various eluates which contained the equivalent amount of 28 mg. of dry weight.

All of the methanolic seed extracts, regardless of the state of dormancy, produced a fluorescent spot on the chromatograms (indicated by circles on the histograms in Fig. 1) which was located usually in or near the region which inhibited the development of the coleoptile sections of wheat. No attempt was made to identify this material. By spraying the chromatogram with specific reagents, the relative positions of the amino acids, organic acids, and sugar were obtained, whereas indole compounds were not detectable in this manner. The sugars extended from the base line up to an Rf value of 0.37, the amino acids ranged from Rf 0.00 to 0.50, while the organic acids were centered in two areas—one near the base line from Rf 0.06 to 0.17 and the other from Rf 0.28 to 0.55. Thus the region which stimulated the elongation of the wheat coleoptiles contained sugars, amino acids, and organic acids, whereas the region of inhibition contained no sugars. The eluates which, at relatively high concentrations, were toxic to the peach embryos also contained amino acids, organic acids, and some sugars. In the bioassays the eluates from near the base line sometimes became turbid within a day or two, and, since this could easily be attributed to bacterial action in the presence of sugars, attempts were made to maintain aseptic conditions for some of the tests with peach. This often delayed but did not overcome eventual discoloration of the ecotyledonized embryos.

Since frequent reference to subsequent seedling development is made in the text, especially below in connection with the quantitative studies of the amino and organic acids, illustrations of the various types are presented in Figures 2 and 3. As shown previously (9), unchilled seeds produced physiological dwarfs (Fig. 2 A) and prechilled seeds produced normal seedlings (Fig. 2 E). The warm temperatures of 20°, 25°, and 30° C. (Fig. 3 F, G, and H) were not adequate for breaking the rest period, whereas a sufficient period of time at the cooler temperatures of 1°, 5°, or 10° C. overcame dormancy (Fig. 3 B, C, and D). Semidwarfs (24) were produced by insufficient chilling at 5° C. (Fig. 2 B) or by being held at



the intermediate temperature of  $15^{\circ}\text{C}$ . (Fig. 3 E). When secondary dormancy (6, pp. 45-48) was imposed on partially-chilled seeds, the seedlings produced (Fig. 2 C) bore no resemblance to the semidwarfed condition (Fig. 2 B) which partially-chilled seeds produced but were, instead, similar to the dwarfs from unchilled seeds. An experiment was designed to determine the time required for the onset of secondary dormancy. Seeds chilled for four weeks at  $5^{\circ}\text{C}$ . were transferred to  $25^{\circ}\text{C}$ . and, with the moist condition maintained, tests were conducted at three- to four-day intervals. Marked changes occurred within three days and by the seventh



FIGURE 2. Seedling development of peach following pretreatment of seeds for (A) 0, (B) 28, (D) 69, and (E) 92 days at  $5^{\circ}\text{C}$ ., (C) 29 days at  $5^{\circ}\text{C}$ ., then 40 days at  $25^{\circ}\text{C}$ . Photographed 45 days after planting in the greenhouse. ( $\times 0.14$ ). No further development occurred in A, B, and C, while growth continued in D and E for an additional 45 days.

day (second test) physiological dwarfs were obtained. Longer periods up to 31 days at  $25^{\circ}\text{C}$ . sustained this condition. Thus, all the seeds in secondary dormancy used in the present studies would have produced dwarfs since at the time of extraction they had been held at  $25^{\circ}\text{C}$ . for periods longer than one week.

In quantitative studies of the 16 amino acids listed in Table I, column 1, the amounts of each increased progressively as the chilling period at  $5^{\circ}\text{C}$ . advanced from 0, 42, and 77 days (columns 2, 3, and 4). The seedlings



produced from these various seeds were dwarfed, semidwarfed, and normal, respectively. However, the mobilization of these materials also occurred at a temperature ( $25^{\circ}\text{C}.$ ) which was not conducive for the production of normal plants (column 7). The amounts which had accumulated when seeds were held for two months at this unfavorable condition were greater than those in the control (column 2). In several instances, such as with arginine and glutamine, the increases were tremendous. Conditions which produced semidwarfs, such as partial chilling (column 3) or inducing secondary dormancy (column 5) or partial chilling after induction of secondary dormancy (column 6), resulted in increases in the various acids. In the latter there were especially large increases of arginine and



FIGURE 3. Seedling development of peach following pretreatment of seeds. Dry control (A), and held for 56 days at  $1^{\circ}$  (B),  $5^{\circ}$  (C),  $10^{\circ}$  (D),  $15^{\circ}$  (E),  $20^{\circ}$  (F),  $25^{\circ}$  (G), and  $30^{\circ}\text{C}.$  (H). Photographed 70 days after planting in the greenhouse. ( $\times 0.10$ ). No further development occurred in A, E, F, G, and H while B, C, and D grew for another 3 weeks.

glutamine and, to a lesser extent, asparagine. The data obtained during germination are also shown in Table I. The unchilled excised seeds, which had been held on moist filter paper in Petri dishes at room temperature in daylight for six days at which time the cotyledons were green and the roots had an average length of approximately 15 mm., were compared with chilled seeds which had been held at  $5^{\circ}\text{C}.$  in the dark for 84 days and had developed at this low temperature a root length of about 18 mm. The latter subsequently produced normal plants while dwarfs were obtained from the former. The data in columns 8 and 9 illustrate what is well known for different seeds, namely, that during germination the amino acids are liberated during proteolysis and that there is a tremendous accumulation

TABLE I

EFFECT OF VARIOUS TEMPERATURE TREATMENTS ON THE FREE AMINO ACIDS  
PRESENT IN PEACH SEEDS

| Amino acid              | Milligrams in 200 seeds |                                           |       |                               |                                                  |                |                                      |       |
|-------------------------|-------------------------|-------------------------------------------|-------|-------------------------------|--------------------------------------------------|----------------|--------------------------------------|-------|
|                         | Dry<br>control*         | Moist seeds at various temperatures, days |       |                               |                                                  |                |                                      |       |
|                         |                         | 5° C.                                     |       | 5° C., 28<br>+<br>25° C., 14* | 5° C., 28<br>+<br>25° C., 14<br>+<br>5° C., 35** | 25° C.,<br>77* | 5° C.                                |       |
|                         |                         | 42**                                      | 77†   |                               |                                                  |                | 0*                                   | 84†   |
|                         |                         |                                           |       |                               |                                                  |                | Germinated<br>at room<br>temperature |       |
| Alanine                 | 2.5                     | 19.2                                      | 27.6  | 14.1                          | 15.7                                             | 7.8            | 7.1                                  | 63.6  |
| Asparagine              | 14.3                    | 47.3                                      | 57.8  | 57.5                          | 65.2                                             | 24.3           | 215.1                                | 222.5 |
| Aspartic                | 3.4                     | 10.5                                      | 20.7  | 16.9                          | 15.5                                             | 11.7           | 12.6                                 | 20.8  |
| Arginine                | 4.1                     | 32.2                                      | 49.5  | 38.5                          | 122.0                                            | 97.6           | 59.9                                 | 100.7 |
| gamma-Amino<br>butyric  | 0.9                     | 4.6                                       | 1.8   | 4.6                           | 3.3                                              | 1.1            | 5.6                                  | 3.9   |
| Glutamic                | 18.9                    | 37.6                                      | 60.6  | 44.1                          | 56.4                                             | 34.9           | 63.9                                 | 66.4  |
| Glutamine               | 3.5                     | 78.0                                      | 242.4 | 52.2                          | 148.6                                            | 108.4          | 300.4                                | 232.1 |
| Glycine                 | 0.5                     | 4.2                                       | 9.2   | 4.4                           | 5.9                                              | 4.1            | 4.6                                  | 15.3  |
| Histidine               | 8.4                     | 9.8                                       | 10.4  | —                             | 10.2                                             | 20.4           | 10.8                                 | 29.3  |
| Leucine,<br>isoleucine  | 2.8                     | 12.6                                      | 21.2  | 3.2                           | 10.7                                             | 7.0            | 8.2                                  | 34.9  |
| Methionine<br>sulfoxide | 3.2                     | 3.2                                       | 8.0   | —                             | 6.0                                              | 10.4           | 4.5                                  | 11.0  |
| Phenylalanine           | 3.5                     | 24.3                                      | 32.7  | 13.5                          | 21.8                                             | 18.0           | 24.0                                 | 40.0  |
| Proline                 | 0.9                     | 31.7                                      | 28.0  | 14.2                          | 14.0                                             | 6.7            | 23.9                                 | 36.9  |
| Serine                  | 0.9                     | 16.6                                      | 29.8  | 12.1                          | 21.3                                             | 14.7           | 22.3                                 | 44.6  |
| Threonine               | 1.5                     | 3.3                                       | 5.5   | 3.4                           | 4.6                                              | 2.2            | 4.4                                  | 8.1   |
| Valine                  | 2.1                     | 4.5                                       | 8.1   | 2.9                           | 7.2                                              | 2.4            | 6.8                                  | 21.8  |

Type of seedlings subsequently obtained: \* physiological dwarf, \*\* semidwarf, † normal.

of the amides—asparagine and glutamine. In respect to the other amino acids there were considerable differences, such as, for instance, alanine and valine where the amounts were much less in the seeds which produced the dwarfs, but it is not known whether any of these differences are significant. Most of these amino acids had previously been identified in various tissues of peach, such as leaves (7), fruit (23), and immature embryos (33).

The organic acids and phosphates also increased in amounts (Table II) when seeds were either partially chilled, adequately chilled, or in secondary dormancy; and, whether or not the seeds had been chilled, tremendous increases occurred during germination. In all of these various extracts the malic acid fraction predominated; however, the great increases in percentage occurred in the phosphates and pyroglutamic acid. Since a quantitative procedure has not as yet been developed for the orthophosphates, the data presented for the phosphates are interpreted merely as incidental information resulting from the organic acid determinations.

In qualitative studies of sucrose, glucose, and fructose (levulose), the

amounts (as indicated by color intensity) of these sugars increased when extracts from the various treatments were compared with those from unchilled control seeds.

Naringenin, the aglycone of naringin, which has been isolated from dormant flower buds of peach and identified by Hendershott and Walker (14), was tested on both the embryos and the seedlings of peach. Over 300 unchilled seeds were soaked either before or after excision for 24 hours at room temperature in solutions of naringenin and naringin which ranged in concentration from 0.5 to 0.0005 mg. per liter. The embryos with cotyledons intact were then transferred to filter paper moistened with the

TABLE II  
EFFECT OF VARIOUS TEMPERATURE TREATMENTS ON THE ORGANIC ACID AND PHOSPHATE CONTENTS OF PEACH SEEDS\*

| Moist seeds at various temperatures, days |        |                  | Subsequent seedling development | Quantity of 0.01N NaOH neutralized, ml.** |             |            |            |
|-------------------------------------------|--------|------------------|---------------------------------|-------------------------------------------|-------------|------------|------------|
| 5° C.                                     | 25° C. | Room temperature |                                 | Pyroglutamic acid†                        | Citric acid | Malic acid | Phosphates |
| 0                                         | 0      | 0                | Dwarf                           | 17.4                                      | 55.7        | 23.5       | 11.1       |
| 70                                        | 0      | 0                | Normal                          | 34.2                                      | 106.9       | 27.8       | 31.0       |
| 38                                        | 14     | 0                | Dwarf                           | 40.0                                      | 110.3       | 39.8       | 27.1       |
| 52                                        | 0      | 0                | Semidwarf                       | 35.5                                      | 137.5       | 44.0       | 41.8       |
| 35                                        | 19     | 0                | Dwarf                           | 39.5                                      | 71.9        | 24.3       | 11.0       |
| 54                                        | 0      | 0                | Semidwarf                       | 44.5                                      | 89.1        | 21.0       | 24.5       |
| 0                                         | 0      | 9††              | Dwarf                           | 89.8                                      | 145.1       | 42.7       | 109.5      |
| 81                                        | 0      | 3††              | Normal                          | 112.0                                     | 178.0       | 51.9       | 151.7      |

\* Gradient elution from anion-exchange resin with 6N formic acid.

\*\* All fractions of a given peak pooled.

† 2-Pyrrolidone-5-carboxylic acid (PCA).

†† Embryos excised and germinated.

respective solutions in Petri dishes, and, upon adequate root development, they were planted in the greenhouse. These concentrations were also tested on unchilled embryos—either ecotyledonized or with only one-half of one cotyledon—by rotating in solution for five days at room temperature in light. The embryos devoid of cotyledons were transferred to nutrient agar, whereas the others were placed on moist filter paper and subsequently planted. Vascular feeding of 35 actively-growing normal seedlings with naringenin (0.5 and 0.005 mg. per liter) and naringin (10 mg. per liter) was attempted over a ten-day period. In another test 72 normal seedlings were sprayed every second or third day with either water or solutions of both naringenin (0.5 mg. per liter) and naringin (10 mg. per liter) until by the 15th day each plant had been sprayed with a total of 14 ml. of a given solution. Naringenin in lanolin (5 mg. per gram) was applied three

times at five-day intervals along the stems, the tips, and new leaves of 50 actively-growing seedlings from the unchilled seeds. Negative results were obtained in all of these various tests which included ample controls in each instance and in which 570 seeds and 160 seedlings were treated.

#### DISCUSSION

Many of the investigators interested in the action of specific substances in breaking the dormancy of buds and tubers were concerned mainly with growth promoters, auxins, etc., until the work of Hemberg (12) on inhibitors. On the other hand, seed-germination inhibitors have been studied for a much longer period of time, but most of the work was not concerned with seeds which require chilling for overcoming the rest period. There have, however, been some studies with apple, *Berberis*, blackberry, *Fraxinus*, and *Sorbus* (1, 15, 16, 30) in which extracts from the seeds in different states of dormancy were tested for inhibitory or stimulatory effects on the development of roots of cress and wheat, *Avena* coleoptile sections, and lettuce seed.

In the present study, bioassays for inhibitors rather than growth-promoting substances were conducted since there was some indication that an inhibitor may be present in the cotyledons of unchilled peach seeds (11). There were substances in the various extracts of peach seeds which were toxic to the peach embryo in relatively high concentrations but ineffectual at lower concentrations. This response did not appear to have any direct relationship with either the presence or the disappearance of dormancy. Wareing and Villiers (30), when testing embryos from the same plant species from which the seed extracts had been obtained, found that while the level of an inhibitor did not change as the dormancy of *Fraxinus* seeds was overcome, there was an increase in concentration of a germination promoter. When investigating dormancy in sycamore, Phillips and Wareing (20) extracted a substance from the buds which promoted the growth of wheat coleoptile, but this response appeared to be due to the presence of sugars. In the data presented above, the materials in the extracts from peach seeds which stimulated the elongation of wheat coleoptiles had been eluted from a region on the chromatogram where the sugars and some of the acids were located. It is of interest that Booth (5) has shown that aqueous extracts of these substances can promote non-hormonal growth. Quantitative data for the acids and phosphates in peach seeds revealed that increments occurred with various treatments, some of which did not break dormancy. In these seeds most of the endosperm is stored in the cotyledons, and, since the embryo was not analyzed separately as was done for tree peony seeds (8), it is assumed that the various levels shown in Tables I and II above reflect changes mainly in the mobilization of the reserve materials. The perennial question of cause versus result persists.



Much more information is needed, especially at the cellular level, before any valid deductions can be made as to the role of any specific substance or substances in regulating metabolic pathways which determine whether the subsequent seedling development will be normal or physiologically dwarfed.

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### Note

## GROWTH REGULANTS IN MAPLE BARK AND FLOWERS<sup>1</sup>

VAS V. ROW<sup>2</sup>

A number of maple (*Acer platanoides* L.) trees near the Institute grounds have formed massive overgrowths along the main trunks. It was thought of interest to determine whether these burls contained growth regulants not found in normal trees. Accordingly tests were performed for the detection of volatile emanations, such as ethylene, and for other growth regulants using paper chromatographic techniques.

Live bark from trees which had developed burls, bark from healthy trees, fresh leaves, and dead bark, loosely packed to the brim in 400-ml. beakers were tested under bell jars for the effect of any volatile emanations on six-week-old tomato (*Lycopersicon esculentum* Mill.) plants. At the end of 18 hours epinastic responses characteristic of those induced by ethylene (1) were noted with the samples of live bark. To test the nature of the emanation the atmosphere above the samples of bark was passed through a dilute solution of bromine which became decolorized within 10 minutes. This suggests the presence of an unsaturated compound such as ethylene (2). Control tests in which the air from the plant parts which had not produced epinasty was passed through bromine solution did not result in any decolorization.

In order to study the presence of other growth regulants in the burls and the bark from healthy trees, about 1000 grams of each were extracted as whole pieces with absolute ethanol for five weeks at  $-7^{\circ}$  C. The extracts were filtered into vacuum flasks and evaporated under reduced pressure at room temperature until only an aqueous fraction remained. This was then filtered and the residue washed with saturated sodium bicarbonate solution (pH 8.6). The filtrate was adjusted to pH 9 and extracted twice with half volumes of ethyl ether. The ether fractions were discarded and the aqueous fractions adjusted to pH 3 and extracted with ether three times. These ether fractions were combined, evaporated in a hood, and the residues taken up in a small volume of isopropanol so that 10 microliters represented 2 grams of the original tissue. Chromatography was carried out on Whatman #1 paper for 18 hours in isopropanol-ammonia-water (80:5:15 v/v) using the ascending technique (4). The chromato-

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grams sprayed with Salkowski reagent showed the presence of indole-3-acetic acid in the bark from healthy trees as well as in the burls. Known samples of IAA were run along with the extracts. When non-sprayed chromatograms were examined under ultraviolet light, additional spots were found in extracts from the burls. Elution of these spots and bioassay with slit pea stem sections revealed growth regulant activity in the extract from the burls, not attributable to IAA. Results are shown in Table I.

Fresh open flowers from Norway maple (*Acer platanoides* L.) and white maple (*A. saccharinum* L.) were frozen in a mixture of solid carbon dioxide and methanol, homogenized in the cold, and extracted with absolute ethanol for 24 hours. Further extraction procedures were similar to those described above. The final ether fractions were subjected to hexane-

TABLE I

RESULTS OF CHROMATOGRAPHY AND BIOASSAY WITH SLIT PEA STEM SECTIONS OF EXTRACTS OF BARK, BURLS, AND FLOWERS OF ACER PLATANOIDES L. AND A. SACCHARINUM L.

| Plant tissue         | Rf values            | Color of spots with indicator | Fluorescence under ultraviolet light | Bioassay activity in slit pea test      |
|----------------------|----------------------|-------------------------------|--------------------------------------|-----------------------------------------|
| Maple bark           | 0.53                 | Purple                        | Blue                                 | Pronounced activity                     |
| Maple burls          | 0.53<br>0.65<br>0.45 | Purple<br>—<br>—              | Blue<br>Blue<br>Blue                 | Pronounced activity<br>Active<br>Active |
| Norway maple flowers | 0.18<br>0.40<br>0.61 | Purple<br>—<br>—              | None<br>Blue<br>Blue                 | Not active<br>Not active<br>Not active  |
| White maple flowers  | 0.40<br>0.20         | Purple<br>—                   | Blue<br>Faint blue                   | Pronounced activity<br>Not active       |

acetonitrile purification (3) in order to remove the large amounts of fatty material. The purified extracts were then chromatographed by the method described for the extracts from the bark. On spraying with Ehrlich reagent, the extract from the flowers of white maple showed a single purple spot at the same Rf value as that of the IAA standard, while the Norway maple flowers showed a single purple spot at Rf 0.18. Non-sprayed chromatograms were also examined under ultraviolet light and spots showing up were eluted and the extracts tested for activity in bioassay using slit pea stem sections. The results are tabulated in Table I.

The presence of three substances with activity in the bioassay in the burls is of interest in view of the accumulating evidence for the occurrence of growth regulants in addition to and other than IAA in plants. Also the fact that extracts of normal bark did not yield the two additional growth regulants indicates a possible relationship to burl formation.

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# INTERRELATIONSHIPS BETWEEN SERUM PROTEIN FRACTIONS ISOLATED BY VARIOUS TECHNIQUES. EVIDENCE FOR AMINO ACID ANLAGEN

RICHARD J. BLOCK<sup>1</sup> AND STEPHEN KELLER<sup>2</sup>

## SUMMARY

The proteins of turtle serum were fractionated by means of diethylaminoethyl-cellulose into eleven components which were characterized by nitrogen, amino acid, and electrophoretic composition.

Although the quantities of amino acids per unit of nitrogen varied by as much as 400 per cent, the molar ratios of eleven pairs of amino acids, in all the protein fractions, were significantly constant at the 1 per cent level and 24 pairs at the 5 per cent level of significance. If these correlations were due to chance, one would expect no more than one pair at the 1 per cent level and 5 to 6 pairs at the 5 per cent level of significance.

The evidence suggests that all the turtle serum proteins, like those of beef, humans, rats, dogs, and chickens, are composed of amino acid anlagen, i.e., a limited number of different peptides, each of which has a relatively constant amino acid content but which differs from each other in amino acid composition.

## INTRODUCTION

Until the middle of the nineteenth century, it was believed that blood serum was composed of a single protein called albumen. Méhu (19) in 1878 showed that proteins could be precipitated from urine apparently unchanged by saturating the urine with ammonium sulfate. Lewith (18) fractionated sera with  $(\text{NH}_4)_2\text{SO}_4$  and reported the presence of two proteins which were later designated globulin and albumin. Following Lewith's work, the use of neutral salts to separate serum proteins into albumin and globulin was widely employed as an aid in clinical diagnosis for it was found that the ratio of albumin to globulin in disease differed from that found in the blood of healthy individuals.

It appears that simply because the albumin/globulin ratio was of clinical value, the idea that there were but two distinct proteins in serum was generally accepted. This was so, even though various investigators including Hardy (15), Sørensen (24), Gróh and Faltin (14), and Block (1, 2) presented evidence that the proteins isolated from serum by different methods appeared to be the result of the method of fractionation employed. This group of investigators suggested that *serum was not composed*

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of just albumin and globulin but that it contained a great number of inter-related proteins.

Hardy (15), from the results of experiments on the behavior of serum in an electrical field (electrophoresis), suggested that serum is not a mixture of independent proteins but that it is composed of a complex protein which breaks down readily into fractions, the composition and properties of which depend upon the degree of dilution and the reagents used. Hardy's ideas were supported by the physicochemical studies of Sørensen (24), and the amino acid analyses of Gróh and Faltin (14), and of Block (1, 2). In order to emphasize the concept that serum may be composed of a multitude of related polypeptides, Block (2, 3) proposed that the term *orosin* be used to designate the total coagulatable protein of serum from which various homogeneous proteins could be derived by physical and chemical manipulations.

What is the experimental evidence for this suggestion? Besides Hardy's (15) work, Sørensen (24) showed that lipide-free crystalline serum albumin is easily and reversibly dissociated into components of different physical and chemical properties. Block (1, 3) found that the lysine content of the globulins prepared by salting out serum with magnesium sulfate, sodium sulfate, ammonium sulfate, or sodium chloride was variable, although the quantity of arginine remained quite constant. Similar discrepancies in the lysine to arginine ratio were also noted in the albumins. On the other hand, when the *orosins* from normal and nephrotic human sera were analyzed for arginine and lysine, it was found that in spite of the large differences in the A/G ratios between the normal and nephrotic sera, the ratio of arginine to lysine was the same as that found in the blood from healthy individuals.

Thus by 1937, serum was generally considered to be composed of two discrete proteins, albumin and globulin, even though a few individuals had suggested otherwise. In that year, Tiselius (25) introduced his new apparatus for the electrophoretic analysis of serum proteins. As a result of this invention and the more recently introduced methods of paper and starch electrophoresis, it is generally believed that there are at least 6 discrete proteins in serum: albumin,  $\alpha_1$ - and  $\alpha_2$ -globulins,  $\beta$ -globulin, and probably several  $\gamma$ -globulins and a few minor components. These electrophoretically separated serum protein fractions are thought to be independent variables in the serum and to be quite unrelated to each other.

With the development of paper and starch electrophoresis (5) and of a successful method of separating serum proteins by column chromatography (20, 23), two further techniques for fractionating serum proteins became available.

Thus, in the last one hundred years the ideas concerning the number of distinct proteins that are present in serum have changed from a single protein (albumen), to two proteins (albumin and globulin), to 6 or more

electrophoretically homogeneous proteins and presently to the idea of a great many discrete proteins. Is there any validity then to the earlier ideas that serum is composed of many interrelated proteins and that the properties of the protein fractions isolated from the serum are dependent in part at least on the reagents used?

The earlier conclusions were and could be criticized on the basis of inadequate methodology. Not only were the methods for separating serum proteins limited to fractional salting out but also only a few amino acids could be estimated with reasonable accuracy. This problem was revived a few years ago by Schultz *et al.* (21, 22) in Philadelphia and by Block and his coworkers (6, 16, 17).

Keller (16) separated bovine serum proteins by fractional precipitation with ammonium sulfate. The ammonium sulfate was introduced into an aqueous solution of the serum proteins by dialysis against salt solutions of increasing ionic strength according to Cohn *et al.* (9). The electrophoretic pattern and the amino acid composition of the fractions were determined and it was shown that certain pairs of amino acids were relatively constant in all the seven fractions.

When human (17) and chicken (6) serum proteins were fractionated on ion-exchange cellulose according to Peterson and coworkers (20, 23), the chemical interrelationships of the various fractions obtained were indicated by the observation that even though the *absolute* quantities of the amino acids per unit of nitrogen varied 2- to 4-fold, the ratio of many pairs of amino acids remained remarkably constant through the spectrum of serum proteins from the  $\gamma$ -globulins to the albumins. The data obtained on the hydrolyzates of the eleven fractions of chicken serum proteins showed that out of 105 possible correlations, 6 pairs (valine:glycine; valine:cystine; lysine:phenylalanine; arginine:threonine; arginine:tyrosine, and aspartic acid:serine) were found to be related at the 1 per cent level and 26 pairs at the 5 per cent level of significance.

Schultz *et al.* (21) separated human, dog, and rat serum proteins into 6 electrophoretically homogeneous fractions by starch electrophoresis. After hydrolysis of the 6 fractions (albumin,  $\alpha_1$ -,  $\alpha_2$ -,  $\alpha_3$ -,  $\beta$ -, and  $\gamma$ -globulins) with 6*N* HCl, these investigators found that the ratio of threonine to aspartic acid was constant in all fractions except in the  $\gamma$ -globulins and that the ratio of glycine plus alanine to aspartic acid was constant in all 6 fractions. Schultz and Delavan (22) also found that partial hydrolyzates of the 6 electrophoretically homogeneous serum protein fractions each yielded at least 8 identical (or very similar) polypeptides on two-dimensional paper chromatograms (electrophoresis in one direction followed by conventional paper chromatography in the second).

In view of the evidence summarized above that all protein fractions obtained from mammalian and avian sera appear to be chemically inter-

related, it was thought of interest to ascertain whether the same phenomenon was present in the serum of another class of animals. As it was not possible to obtain sera from various reptiles, the studies were restricted to the blood of a single species of sea turtle (*Chelonia mydis* L.).

#### EXPERIMENTAL METHODS

The proteins of turtle serum were separated by means of paper electrophoresis, using 0.075*M* barbital buffer at pH 8.6 for 16 hours (5). No albumins were found.<sup>3</sup> However, in order to ascertain if turtle serum was truly devoid of albumins, the serum was half-saturated with ammonium sulfate and the resulting filtrate subjected to paper electrophoresis. All the protein remaining in solution at one-half saturation with ammonium sulfate moved to the usual albumin position at pH 8.6.

The proteins of turtle serum were separated next by chromatography on DEAE-SF-cellulose (diethylaminoethylcellulose prepared from Solka-floc) (20, 23). Fifty ml. of serum were placed on a 2.2×25 cm. column of DEAE-SF-cellulose, in the presence of a 0.01*M*, pH 8.0, sodium phosphate buffer. The proteins were eluted from the column by a single gradient of pH and ionic strength, as employed by Fahey *et al.* (10). This gradient was produced by introducing 0.3*M* NaH<sub>2</sub>PO<sub>4</sub> from a one-liter Erlenmeyer flask into two liters of a 0.01*M* sodium phosphate buffer at pH 8.0. The latter was constantly stirred and fed into the column by means of a siphon. The various proteins eluted from the cellulose were detected by their ultraviolet absorption at 280 mμ, and the eluates comprising each of the protein peaks were dialyzed free of salts and lyophilized. The fractions were analyzed for total nitrogen and electrophoretic components. Eight of the protein fractions from the column, as well as the euglobulin which precipitated during dialysis against water, were analyzed for their constituent amino acids by quantitative paper chromatography (4, 5).

#### RESULTS

Figure 1 shows the protein peaks that were eluted from the DEAE-cellulose column. Peak CD was subdivided as indicated.

The nitrogen values given in Table I show that a number of the fractions (A, G, H, I, J) contained approximately an equal weight of non-protein materials, presumably lipides and carbohydrates. These nonprotein substances in the protein fractions are compensated for in the calculations shown on Table II because all the amino acid values given in this table are calculated in millimoles per 1.6 g. of nitrogen. Although only 3 of the

<sup>3</sup> Zweig and Crenshaw (28) and Cohen and Strickler (8) have reported the absence of a component in turtle serum which moves on paper electrophoresis to the albumin position. They concluded from their findings that turtle serum proteins do not contain an albumin-like fraction. Frieden (12) has made a similar observation on swamp frog serum.

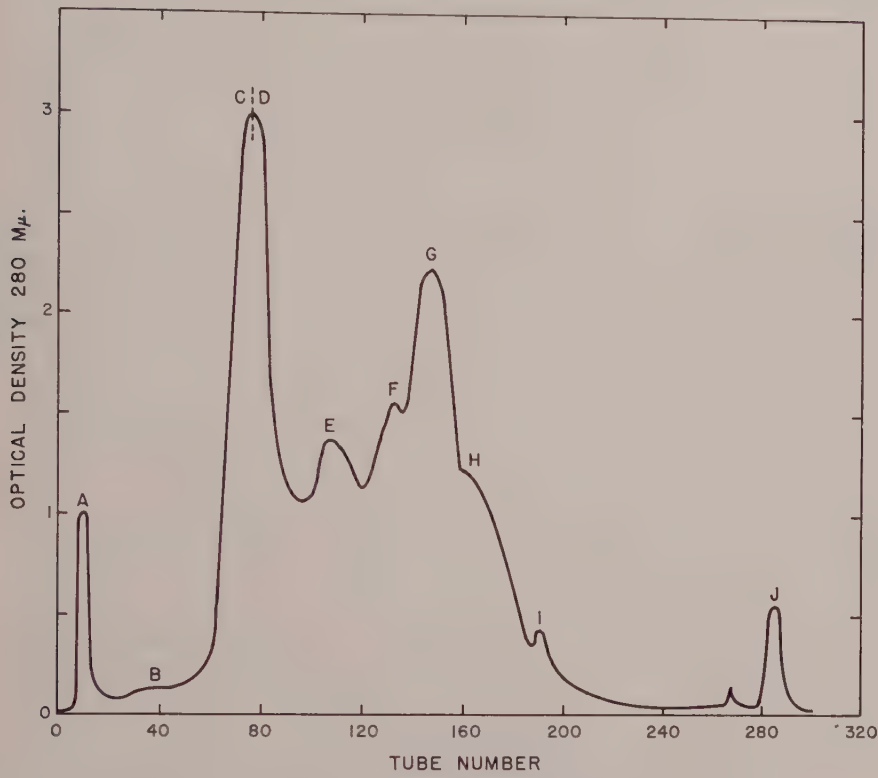


FIGURE 1. Distribution of turtle serum proteins on elution from a DEAE-SF-cellulose column by sodium phosphate buffer.

TABLE I  
COMPOSITION AND NITROGEN CONTENT OF THE TURTLE SERUM  
PROTEIN FRACTIONS SEPARATED ON DEAE-CELLULOSE

| Fraction    | Per cent nitrogen* | Globulin components** |           |              | $\alpha_1$ | Albumins** |
|-------------|--------------------|-----------------------|-----------|--------------|------------|------------|
|             |                    | $\gamma$ -            | $\beta$ - | $\alpha_2$ - |            |            |
| A           | 6.7                |                       | 100       |              |            |            |
| B           | —                  |                       | 100       |              |            |            |
| C           | 13.9               | 100                   |           |              |            |            |
| D           | —                  | 59                    |           | 8            |            | 33         |
| E           | 11.1               | 23                    | 15        | 11           |            | 51         |
| F           | 13.2               | 15                    | 10        | 36           | 9          | 30         |
| G           | 9.3                |                       | 23        | 38           |            | 39         |
| H           | 8.7                |                       | 53        |              |            | 47         |
| I           | 8.4                |                       | 17        | 20           |            | 63         |
| J           | 9.9                |                       | 100       |              |            |            |
| Euglobulins | 13.8               | 44                    | 56        |              |            |            |

\* Uncorrected for moisture and ash.  
\*\* Per cent of each fraction.



TABLE II  
AMINO ACID COMPOSITIONS OF THE TURTLE SERUM PROTEIN FRACTIONS

| Amino acid             | Millimoles of amino acid/1.6 g. of nitrogen for the protein fractions |      |      |      |      |      |      |      | Euglobulin |
|------------------------|-----------------------------------------------------------------------|------|------|------|------|------|------|------|------------|
|                        | A                                                                     | C    | E    | F    | G    | H    | I    | J    |            |
| Arginine               | 2.6                                                                   | 3.0  | 3.6  | 2.9  | 3.4  | 3.7  | 2.3  | 3.6  | 3.6        |
| Histidine              | 1.1                                                                   | 1.8  | 2.9  | 2.2  | 2.4  | 2.4  | 1.5  | 2.4  | 2.2        |
| Lysine                 | 3.1                                                                   | 5.1  | 7.6  | 5.4  | 5.5  | 5.1  | 3.6  | 4.9  | 5.6        |
| Tyrosine               | 1.3                                                                   | 2.8  | 3.2  | 2.3  | 3.6  | 3.9  | 2.3  | 2.3  | 3.0        |
| Cystine                | 1.1                                                                   | 1.8  | 3.9  | 1.8  | 2.2  | 1.5  | 1.8  | 2.1  | 2.6        |
| Methionine             | 0.5                                                                   | 0.9  | 1.7  | 1.2  | 1.5  | 1.2  | 1.0  | 0.9  | 1.5        |
| Serine                 | 5.3                                                                   | 6.3  | 5.8  | 5.2  | 4.1  | 3.9  | 2.9  | 7.0  | 5.3        |
| Threonine              | 3.6                                                                   | 6.0  | 6.2  | 5.1  | 5.2  | 4.4  | 3.8  | 5.7  | 5.7        |
| Leucine and isoleucine | 4.3                                                                   | 4.5  | 6.6  | 6.3  | 5.7  | 5.9  | 5.1  | 4.7  | 5.4        |
| Valine                 | 2.9                                                                   | 4.0  | 4.0  | 4.0  | 5.2  | 5.2  | 3.2  | 3.7  | 4.3        |
| Glutamic acid          | 6.9                                                                   | 11.4 | 19.8 | 15.0 | 16.5 | 14.8 | 12.8 | 16.6 | 16.0       |
| Aspartic acid          | 5.5                                                                   | 5.8  | 6.9  | 6.2  | 7.1  | 7.2  | 5.4  | 7.9  | 8.0        |
| Glycine                | 4.5                                                                   | 5.7  | 5.2  | 4.5  | 4.8  | 4.8  | 3.8  | 5.8  | 6.3        |
| Alanine                | 5.3                                                                   | 6.9  | 8.2  | 7.0  | 5.5  | 6.1  | 4.0  | 6.5  | 4.4        |
| Phenylalanine*         | —                                                                     | 2.7  | 3.8  | —    | 3.1  | 3.3  | 3.1  | —    | 3.4        |

\* Omitted from calculations.

9 fractions for which amino acid analyses were made were electrophoretically homogeneous, the amino acid values were analyzed statistically in order to determine whether there were any significant correlations between the amino acids in the 10 protein fractions. Out of a possible 105 combinations, eleven pairs were found to have correlation coefficients significant at the 1 per cent level and 24 pairs at the 5 per cent level. Some of the results of this statistical treatment are presented in Table III. Of the 14 amino acids for which an analysis was made, 12 of them could be paired with at least one other amino acid. Serine, alone, could not be paired. An analogous finding has been reported previously for the chicken serum pro-

TABLE III  
REGRESSION ANALYSIS OF TWELVE PAIRS OF AMINO ACIDS

| Amino acid pair | Regression equation   | Product moment correlation coefficient | Rank correlation coefficient |
|-----------------|-----------------------|----------------------------------------|------------------------------|
| Val:Tyr         | Val = 1.85 + 0.78 Tyr | 0.95                                   | 0.95                         |
| Glu:His         | His = 0.24 + 0.13 Glu | 0.81                                   | 0.94                         |
| Arg:His         | Arg = 1.80 + 0.65 His | 0.68                                   | 0.91                         |
| Leu:Met         | Leu = 2.86 + 2.12 Met | 0.98                                   | 0.88                         |
| Asp:Arg         | Asp = 2.61 + 2.82 Arg | 0.78                                   | 0.86                         |
| Ala:Lys         | Ala = 1.00 + 1.00 Lys | 0.79                                   | 0.85                         |
| Glu:Arg         | Glu = 0.66 + 4.69 Arg | 0.70                                   | 0.83                         |
| Glu:Cys         | Glu = 7.09 + 3.42 Cys | 0.84                                   | 0.83                         |
| Glu:Asp         | Glu = 1.17 + 2.06 Asp | 0.71                                   | 0.82                         |
| Asp:His         | Asp = 3.47 + 1.46 His | 0.67                                   | 0.80                         |
| Thr:Lys         | Thr = 2.10 + 0.58 Lys | 0.79                                   | 0.80                         |
| His:Cys         | His = 0.92 + 0.53 Cys | 0.83                                   | 0.78                         |

teins (6) except in this case glutamic acid was the only amino acid that could not be paired with at least one other.

Because statistical treatment appeared to indicate that only one amino acid was present both in chicken and in turtle serum which could not be paired with at least one other amino acid, a similar type of statistical treatment was carried out as a blind analysis on the amino acids present in 8 proteins (bovine serum albumin, human hemoglobin, crystalline egg albumin, insulin,  $\beta$ -lactoglobulin, actin, gelatin, and ribonuclease). The data were presented to the statistician (D. W. Miller), as had been done previously, that is, as the amino acids in fractions 1 to 8 inclusive. Of 105 pairs, 5 pairs were significantly related at the 5 per cent level and 3 pairs had a significant negative correlation. This distribution is that expected by chance. The results of the statistical treatment of the amino acids in the 8 unrelated proteins give greater confidence to the thesis reported here.

#### DISCUSSION

Although the proteins in turtle serum could be readily separated on DEAE-cellulose, it is interesting that there is a significant difference in the chromatographic behavior between mammalian and avian or reptilian sera. The first 6 protein fractions to appear off the column when human serum is chromatographed are  $\gamma$ -globulins (17). These are followed by  $\beta$ -globulins. However, in the case of both turtle and chicken (6) sera, the  $\beta$ -globulins are eluted before the  $\gamma$ -globulins. These differences may be due to variations in the distribution of charge or to the molecular size of the  $\gamma$ - and  $\beta$ -globulins in the species tested.

Amino acid analysis of the protein fractions of turtle serum showed some large differences in composition between electrophoretically related proteins. Although fractions A and J were both  $\beta$ -globulins, they showed variations in amino acid composition from 10 to 100 per cent. Similar differences were noted in human and chicken sera for various  $\gamma$ - and  $\beta$ -globulins (6, 17). Thus  $\gamma$ - and  $\beta$ -globulins may possess a number of polypeptides which differ in their amino acid composition.

The finding, that the various protein fractions of turtle serum are composed of many pairs of amino acids which exist in approximately constant molar ratio,<sup>4</sup> is in agreement with the relationships uncovered for the proteins of mammalian and avian sera (6, 16, 17, 21). These interrelationships taken together with the findings of Schultz and Delavan (22) suggest the presence of peptides of constant over-all composition in the various proteins of a given serum. This may have little to do with any of the

<sup>4</sup> The criticism can be made that the constant amino acid ratio seen in fractions E to I inclusive may be the result of the presence of albumins in all these fractions. This objection is probably not valid. Purified albumins contain more cystine than any of the globulins, yet fraction E with 51 per cent albumin contained 3.9 millimoles of cystine per 1.6 g. of N, while fraction I with 63 per cent albumin contained only 1.8 millimoles.

functional activities of the different proteins, but rather may indicate a slow molecular evolution of the serum proteins from a common precursor protein (6). However, the amino acid pairs do vary in absolute quantity. The serum proteins, therefore, appear to be composed of different amounts of each of these amino acid groups, anlagen, and the various properties of the serum proteins may be a function of the amount of each of the anlagen present. These findings also imply that the serum protein-forming templates themselves have structural similarities. Such a condition is not without precedent, for it has been shown that the pituitary hormones have structural similarities, probably produced by templates having common nucleic acid structures (13, 26).

The situation regarding the synthesis of plasma proteins is not clear. Yuile *et al.* (27) have demonstrated that cellular protein synthesis occurring in dogs, after intravenous administration of  $C^{14}$ -labeled plasma proteins, is accompanied by very little nitrogen excretion and these results have been interpreted as indicating that the plasma proteins are used after only partial catabolism to the peptide level. It has also been shown (7) that labeled plasma proteins enter ascites cells and are used for the synthesis of cell protein, presumably being broken down only to the peptide level. On the other hand, Fleischer *et al.* (11) say "Our results show

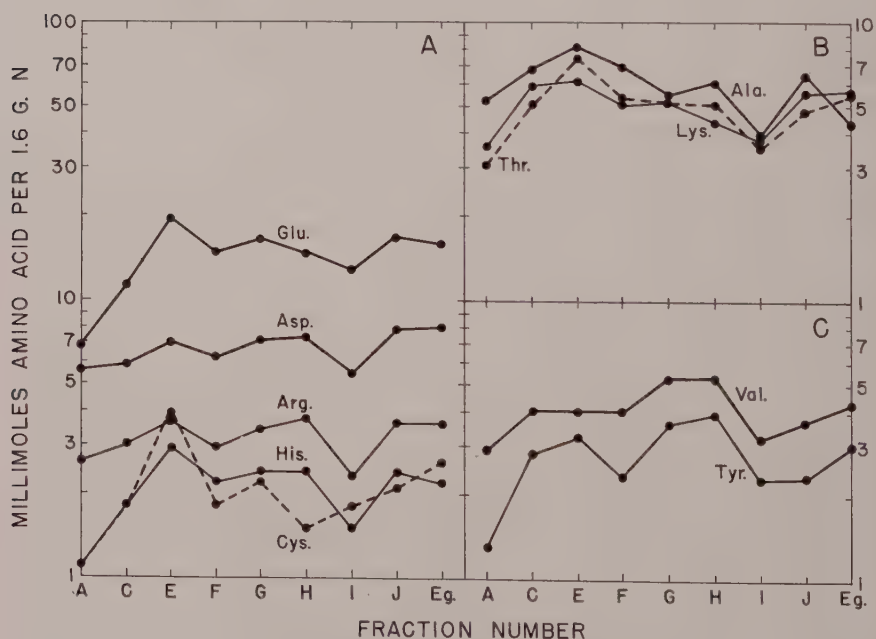


FIGURE 2. Distribution of some interrelated amino acids in the protein fractions of turtle serum.

that conversion of serum proteins into tissue proteins involves complete breakdown to the amino acid stage."

Figure 2 illustrates some of the amino acid relationships in the proteins of turtle serum. The group of five amino acids related at the 1 per cent level (cystine, histidine, arginine, aspartic acid, and glutamic acid) (Table III) may be seen in Figure 2 A to vary in a dependent manner. The threonine, lysine, alanine group is graphed in Figure 2 B, while valine and tyrosine are shown in Figure 2 C. Several other relationships exist but at a lower level of significance. Cystine appears to be of special importance because of its constant molar ratio to a relatively large number of amino acids. On the other hand, serine does not appear to be associated with any other amino acid for which analyses were made. There are some pairs of amino acids which occur in a constant ratio in both turtle and avian sera, e.g., aspartic acid:arginine; cystine:aspartic acid, and valine:tyrosine. Furthermore, the actual levels of each amino acid in the comparable proteins of human, avian, and turtle sera are quite similar.

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# STORAGE OF SEEDS OF *LOBELIA CARDINALIS* L.

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## SUMMARY

Seeds of *Lobelia cardinalis* L. were stored in sealed glass vials with moisture contents of 6.9 and 4.7 per cent in atmospheres of air, oxygen, carbon dioxide, nitrogen, and partial vacuum for periods up to 25 years, at laboratory temperature, and at 5°, and -5° C. They were also stored in open containers at these temperatures.

Viability was lost more rapidly in the laboratory than at the lower temperatures, and at the higher moisture content than at the lower. At laboratory temperature in open storage or in sealed vials in air, viability was reduced from 73 to 38 and 52 per cent respectively after one year of storage with rapid deterioration thereafter. Deterioration was even more rapid in an atmosphere of oxygen, while storage in carbon dioxide, nitrogen, or a partial vacuum extended the life of the seeds held in the laboratory for as much as 6 or 8 years.

Life span in open storage at 5° C. under very humid conditions was as short as that in the drier atmosphere of the laboratory, while seeds in open storage at -5° C. survived for more than 12 years. There were no differences in the response of the seeds to atmospheres of air, oxygen, carbon dioxide, nitrogen, or partial vacuum at the two low temperatures in 25 years of storage.

## INTRODUCTION

The species of *Lobelia* are prized as ornamental flowers but very little is known about the life span of their seeds. Apparently some of them retain viability for many years since Aberconway (1) reported the appearance of an unidentified species in a cultivated garden plot where no *Lobelia* had been grown for 32 years.

To the best of the author's knowledge no one had made controlled tests on the storage of any species of *Lobelia* so a series of experiments was begun with the 1934 crop of seed from the cardinal flower, *Lobelia cardinalis* L. These seeds have been tested periodically for viability after storage under different conditions for the past 25 years. The data obtained are summarized in this paper.

## MATERIALS AND METHODS

Seeds were harvested in the fall of 1934 from a field plot and were placed under special storage conditions in November of that year. At the time the experiments were started the seeds were dried to constant weight at 80° to 85° C. and found to have 6.9 per cent moisture (dry weight basis). The seeds were divided into two equal lots after thoroughly mixing them and the moisture content of one lot was reduced to 4.7 per cent by holding them over calcium oxide in a desiccator for 9 days. The two lots were then subdivided into samples of approximately 1,000 seeds each and placed in

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individual glass receptacles of about 2-ml. capacity made from a tube by sealing one end and drawing the other to a fine neck. This provided about 10 volumes of air or other gas for each volume of seed because the seeds were small. The air in the tubes was displaced with carbon dioxide, oxygen, nitrogen, or fresh air, or evacuated by an oil exhaust pump to create a partial vacuum before sealing the necks of the tubes by heat.

Sufficient tubes (30) with each gas at each moisture content were stored at ambient laboratory temperature, 5° C., and -5° C., to permit periodic removal of a tube from each series and testing of the seeds for viability. As a standard control at each storage temperature one large lot of seeds was held in an open bottle where there would be free exchange of moisture and gases with the atmosphere.

Viability was measured by the ability of the seed lot to produce seedlings in soil in the greenhouse following a pretreatment period of two months at 5° C. after planting. Other germination tests were made and will be described below.

Tests have been made after 1, 1.5, 2, 2.5, 3, 4, 6, 8, 11.5, 12.5, 14.5, 16, 19, 20, 22, 24, and 25 years of storage.

## EXPERIMENTAL RESULTS

### GERMINATION TESTS

Initial germination tests were carried out with duplicates of 100 seeds each on moist filter paper in Petri dishes at constant temperatures of 15°, 20°, 25°, 30°, and 35° C. and at daily alternating temperatures of 10° to 20° C., 10° to 30° C., 15° to 30° C., and 20° to 30° C. In the case of alternating temperatures, cultures were left at the lower temperature for 16 hours and at the higher temperature for 8 hours daily Monday to Friday inclusive. On Saturday and Sunday they were kept at the lower temperature. Tests were also conducted in dark and light in the laboratory. Best germination was obtained at a daily alternating temperature of 10° to 30° C. and in the light in the laboratory (63 and 45 per cent respectively).

Soil tests were also conducted, in which the seeds were planted on top of a fine mixture of soil, sand, and granulated peat moss in equal parts, in 4-inch pots. The pots were then placed in water, watered thoroughly from below, covered with glass, and placed in a bed of granulated peat moss. Some of the pots were placed directly in the greenhouse while others were pretreated for 1, 2, and 3 months at 1°, 5°, and 10° C., after which they were transferred to the greenhouse to germinate. Good seedling production (up to 73 per cent) was secured after pretreatment at 5° or 10° C. for 2 or 3 months, while seeds placed in the greenhouse without pretreatment gave about 1 per cent germination.

The initial germination tests were made in November since that is the time the seeds were received and stored. Subsequent tests were made in

TABLE I

GERMINATION OF SEEDS OF *LOBELIA* ON MOIST FILTER PAPER IN PETRI DISHES IN LIGHT IN THE LABORATORY AND IN SOIL IN THE GREENHOUSE WITH AND WITHOUT PRETREATMENT FOR 2 MONTHS AT 5° C.

| Storage conditions |            |                 | Germination (%) after storage |    |         |    |                         |    |         |    |
|--------------------|------------|-----------------|-------------------------------|----|---------|----|-------------------------|----|---------|----|
| Temp.              | Con-tainer | Gas             | 12 Months (test in Nov.)      |    |         |    | 30 Months (test in May) |    |         |    |
|                    |            |                 | On filter paper               |    | On soil |    | On filter paper         |    | On soil |    |
|                    |            |                 | a*                            | b* | a       | b  | a                       | b  | a       | b  |
| Labora-tory        | Open       | Air             | 20                            | 55 | 6       | 38 | 50                      | 41 | 49      | 27 |
|                    | Sealed     | Air             | 71                            | 54 | 2       | 52 | 38                      | 40 | 47      | 20 |
|                    |            | O <sub>2</sub>  | 33                            | 23 | 2       | 16 | 1                       | 3  | 2       | 1  |
|                    |            | CO <sub>2</sub> | 69                            | 79 | 1       | 77 | 69                      | 71 | 82      | 54 |
|                    |            | N               | 71                            | 84 | 5       | 81 | 75                      | 69 | 89      | 53 |
| -5° C.             |            | Vac.            | 82                            | 77 | 22      | 90 | 67                      | 77 | 74      | 70 |
|                    | Open       | Air             | 90                            | 88 | 8       | 92 | 80                      | 77 | 96      | 62 |
|                    | Sealed     | Air             | 79                            | 60 | 14      | 94 | 62                      | 83 | 81      | 74 |
|                    |            | O <sub>2</sub>  | 63                            | 89 | 4       | 55 | 75                      | 64 | 91      | 69 |
|                    |            | CO <sub>2</sub> | 79                            | 67 | 4       | 92 | 82                      | 93 | 90      | 59 |
|                    |            | N               | 80                            | 64 | 5       | 77 | 78                      | 86 | 87      | 44 |
|                    |            | Vac.            | 40                            | 78 | 8       | 76 | 67                      | 77 | 87      | 66 |

\* a = No pretreatment; b = pretreated for 2 months at 5° C. after planting.

May after 1.5 and 2.5 years of storage. It was noted that seeds planted in soil in the greenhouse in May germinated well without low-temperature pretreatment. This is shown in Table I, in which the results of the 1-year test are compared with those of the 2.5-year test. This superiority of germination in spring plantings is probably due to the amount of light, since these seeds are known to be light-favored. Greenhouse day temperatures at that time also approximate the 30° C. found beneficial in alternating temperature tests.

Data from tests with seeds pretreated at low temperature and germinated on moist filter paper in Petri dishes in light in the laboratory showed no beneficial effect of the low temperature pretreatment in either November or May planting (Table I).

In order to standardize the viability tests and to prevent as much variation as possible from test to test, the standard method of planting 100 seeds in soil in four replicate pots, two of which were placed in the greenhouse immediately and two of which were kept for 2 months at 5° C. before transfer to the greenhouse was adopted. Seedling production after low-temperature pretreatment was used as a measure of comparative viability of the different lots.



## VIABILITY AFTER STORAGE

Seedling production in soil in the greenhouse after pretreatment for two months at 5° C. after different periods of storage up to 25 years is shown in Table II.

Seeds containing 6.9 per cent moisture lost their viability rather quickly in the laboratory in open containers or in sealed containers in an atmosphere of air. Viability was reduced to 38 and 52 per cent respectively under these conditions after one year of storage, with rapid deterioration thereafter. Death came even more rapidly in an atmosphere of oxygen, while carbon dioxide, nitrogen, or a partial vacuum extended the life of the seeds for as much as 6 or 8 years. Reduction of the moisture content to 4.7 per cent resulted in an extension of germination capacity under all condi-

TABLE II

SEEDLING PRODUCTION IN THE GREENHOUSE FROM STORED SEEDS OF LOBELIA PLANTED IN SOIL AND PRETREATED FOR 2 MONTHS AT 5° C.

| Storage conditions    |           |                 | Per cent seedling production after years of storage |    |    |    |    |    |      |      |      |    |    |    |    |    |    |  |  |
|-----------------------|-----------|-----------------|-----------------------------------------------------|----|----|----|----|----|------|------|------|----|----|----|----|----|----|--|--|
| Temp.                 | Container | Gas             | 1                                                   | 2  | 3  | 4  | 6  | 8  | 11.5 | 12.5 | 14.5 | 16 | 19 | 20 | 22 | 24 | 25 |  |  |
| 6.9 per cent moisture |           |                 |                                                     |    |    |    |    |    |      |      |      |    |    |    |    |    |    |  |  |
| Laboratory            | Open      | Air             | 38                                                  | 20 | 13 | 2  | 0  | —  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
|                       | Sealed    | Air             | 52                                                  | 12 | 22 | 8  | 6  | 3  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
|                       |           | O <sub>2</sub>  | 16                                                  | 5  | 1  | 1  | 0  | —  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
|                       |           | CO <sub>2</sub> | 77                                                  | 71 | 30 | 27 | 42 | 6  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
| N                     |           | 81              | 20                                                  | 62 | 67 | 58 | 70 | 1  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
| Vac.                  | 90        | 45              | 73                                                  | 34 | 50 | 37 | 1  | —  | —    | —    | —    | —  | —  | —  | —  | —  |    |  |  |
| 5° C.                 | Open      | Air             | 63                                                  | 6  | 1  | 0  | 0  | —  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
|                       | Sealed    | Air             | 73                                                  | 57 | 60 | 68 | 77 | 91 | 87   | 96   | 87   | 59 | 43 | 73 | 65 | 57 | 58 |  |  |
|                       |           | O <sub>2</sub>  | 76                                                  | 70 | 54 | 32 | 65 | 90 | 85   | 97   | 88   | 66 | 65 | 65 | 59 | 44 | 52 |  |  |
|                       |           | CO <sub>2</sub> | 91                                                  | 52 | 54 | 68 | 47 | 87 | 87   | 91   | 83   | 71 | 53 | 59 | 30 | 35 | 52 |  |  |
| N                     |           | 61              | 51                                                  | 62 | 69 | 69 | 89 | 81 | 87   | 88   | 65   | 58 | 59 | 68 | 63 | 54 |    |  |  |
| Vac.                  | 85        | 53              | 44                                                  | 52 | 77 | 87 | 84 | 86 | 86   | 50   | 42   | 48 | 45 | 30 | 62 |    |    |  |  |
| -5° C.                | Open      | Air             | 92                                                  | 55 | 72 | 76 | 59 | 80 | 57   | 46   | 23   | 14 | 1  | 1  | —  | —  | —  |  |  |
|                       | Sealed    | Air             | 94                                                  | 75 | 66 | 70 | 74 | 92 | 90   | 93   | 75   | 49 | 57 | 69 | 49 | 35 | 45 |  |  |
|                       |           | O <sub>2</sub>  | 55                                                  | 55 | 50 | 60 | 56 | 96 | 74   | 94   | 85   | 52 | 48 | 31 | 71 | 41 | 58 |  |  |
|                       |           | CO <sub>2</sub> | 92                                                  | 64 | 64 | 60 | 57 | 91 | 92   | 95   | 92   | 55 | 66 | 62 | 27 | 45 | 50 |  |  |
| N                     |           | 77              | 27                                                  | 60 | 65 | 78 | 90 | 85 | 95   | 81   | 56   | 26 | 66 | 54 | 21 | 43 |    |  |  |
| Vac.                  | 76        | 48              | 45                                                  | 64 | 76 | 95 | 84 | 97 | 76   | 60   | 44   | 49 | 50 | 48 | 62 |    |    |  |  |
| 4.7 per cent moisture |           |                 |                                                     |    |    |    |    |    |      |      |      |    |    |    |    |    |    |  |  |
| Laboratory            | Sealed    | Air             | 72                                                  | 33 | 58 | 33 | 28 | 48 | 17   | 24   | 9    | 4  | —  | —  | —  | —  | —  |  |  |
|                       |           | O <sub>2</sub>  | 68                                                  | 49 | 29 | 10 | 10 | 0  | —    | —    | —    | —  | —  | —  | —  | —  | —  |  |  |
|                       |           | CO <sub>2</sub> | 87                                                  | 58 | 55 | 53 | 76 | 95 | 75   | 80   | 58   | 44 | 31 | 16 | 4  | 6  | 4  |  |  |
|                       |           | N               | 93                                                  | 68 | 59 | 70 | 60 | 92 | 55   | 75   | 52   | 41 | 38 | 24 | 7  | 2  | 1  |  |  |
| Vac.                  | 81        | 63              | 76                                                  | 66 | 58 | 88 | 73 | 87 | 66   | 67   | 21   | 11 | 3  | 4  | 0  | —  |    |  |  |
| 5° C.                 | Sealed    | Air             | 79                                                  | 71 | 65 | 62 | 73 | 87 | 92   | 93   | 75   | 71 | 66 | 72 | 68 | 95 | 58 |  |  |
|                       |           | O <sub>2</sub>  | 94                                                  | 69 | 52 | 57 | 82 | 93 | 67   | 92   | 67   | 64 | 72 | 84 | 48 | 71 | 59 |  |  |
|                       |           | CO <sub>2</sub> | 60                                                  | 55 | 37 | 73 | 58 | 86 | 3    | 92   | 71   | 67 | 43 | 59 | 50 | 47 | 49 |  |  |
|                       |           | N               | 79                                                  | 80 | 79 | 50 | 36 | —  | 82   | 94   | 65   | 78 | 63 | 64 | 45 | 46 | 57 |  |  |
| Vac.                  | 74        | 70              | 64                                                  | 64 | 48 | 93 | 77 | 86 | 64   | 67   | 33   | 46 | 50 | 56 | 51 | —  |    |  |  |
| -5° C.                | Sealed    | Air             | 43                                                  | 57 | 51 | 68 | 67 | 83 | 75   | 95   | 91   | 79 | 68 | 73 | 45 | 60 | 64 |  |  |
|                       |           | O <sub>2</sub>  | 87                                                  | 40 | 69 | 53 | 78 | 89 | 95   | 91   | 81   | 85 | 7  | 68 | 44 | 51 | 55 |  |  |
|                       |           | CO <sub>2</sub> | 64                                                  | 51 | 63 | 43 | 65 | 85 | 66   | 94   | 83   | 60 | 41 | 63 | 43 | 50 | 51 |  |  |
|                       |           | N               | 88                                                  | 48 | 68 | 78 | 46 | 93 | 87   | 91   | 87   | 72 | 46 | 75 | 58 | 51 | 44 |  |  |
| Vac.                  | 84        | 31              | 59                                                  | 58 | 64 | 89 | 81 | 91 | 79   | 68   | 52   | 58 | 73 | 47 | 59 | —  |    |  |  |

tions of laboratory storage, but, again, carbon dioxide, nitrogen, and partial vacuum storage proved superior to air storage and an oxygen atmosphere proved most deleterious.

It was to be expected that low-temperature storage would increase the life span, and this was found to be true except for open storage at 5° C. where the air was saturated with moisture. The air of the room at approximately -5° C. was also moisture-saturated, but the extra cold permitted retention of viability in open storage for a longer period than at 5° C.

It will be noted that there was considerable variation in the seedling production from year to year in spite of efforts to control the germination conditions. It is believed that this is due, at least in part, to the error of sampling these small seeds, though only plump, apparently filled, seeds were counted from the storage tubes.

To determine whether there were differences in the response of the seeds to atmospheres of carbon dioxide, nitrogen, or partial vacuum in the laboratory, or to air, oxygen, carbon dioxide, nitrogen, or vacuum at the two low temperatures, germination percentages were converted to equivalent angles and an analysis of variance was conducted. The only significant differences shown in these cases were those resulting from the increased period in storage.

#### DISCUSSION

The role of low temperature and low moisture content in the extension of life span has been reported extensively for many kinds of seeds. However, less experimental work has been done on the effect of different gases in contact with the seeds. Simpson (14) described a detailed experiment on cottonseed in various gases under controlled temperature and moisture. The gases used were air, oxygen, carbon dioxide, and nitrogen. Examination of the samples was made every 6 months for 3.5 years, and lots with 7 per cent moisture at 70° F. were tested after 8 and 10 years of storage. The author concluded that, within the limits of his experiment, the initial atmosphere in the storage chamber had no effect on the longevity of cottonseed, and that free oxygen was not essential to the continuous life processes of the seed. Sayre (13) reached the same conclusion as a result of sealing corn for five years in air, oxygen, carbon dioxide, and nitrogen. However, Sayre found differences in corn in the various atmospheres, the loss in viability being more pronounced in oxygen and nitrogen atmospheres than in air or carbon dioxide. Sayre (13) stated that respiration may speed deterioration and any condition which will decrease respiration will extend the life of the seed. The reason for the harmful effect of the nitrogen atmosphere on stored corn is not clear. No such effect of nitrogen was found in the present tests with *Lobelia* seeds.

Busse (7) stored poplar seeds in air and in partial vacuum and found

the life span increased by reducing the air pressure. He believed this was due to the injurious action of the oxygen in the air. Guillaumin (8) demonstrated by an experiment lasting 12 years that vacuum was a means of preserving the viability of grains. An atmosphere devoid of oxygen was as good as a vacuum. Sampietro (12) found that nitrogen extended the life of rice seeds in storage, and that this gas was better than carbon dioxide, air, or vacuum. Air and carbon dioxide had the same effect on viability of rice in storage according to Kondo and Okamura (10).

Seeds of the American elm stored in an atmosphere of oxygen lost their viability more quickly than when sealed in air. When moisture or temperature conditions were unfavorable, a partial vacuum extended the life of these seeds (4). Some benefits of a lessened oxygen supply may have been experienced by conifer seeds under certain storage conditions (3), but aster and verbena seeds were not improved by a partial vacuum under the conditions used by Barton (5).

It seems certain that most seeds can carry on the minimum metabolic activity necessary for retention of viability without an outside supply of oxygen. Furthermore, oxygen seems to be actually harmful in cases of high seed moisture content or storage at high temperature. It is to be expected that an extended storage period, beyond 25 years in the case of *Lobelia*, would bring about more rapid deterioration under oxygen than nitrogen or vacuum even at a temperature below freezing, an effect evident at a much earlier period in laboratory storage.

Further evidence of the harmful effect of oxygen on seeds is to be found in the enhancement of soaking injury in the presence of this gas (6). Also X-rays are more injurious when the seeds are treated with oxygen either before or after irradiation. Oxygen in the storage chamber increases and carbon dioxide and nitrogen decrease the chromosomal aberrations which accompany aging in seeds (2, 9, 11, 15, and others).

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# LIFE-SPAN OF FROST-DAMAGED CORN SEEDS

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## SUMMARY

Five different lots of frost-damaged hybrid sweet corn (*Zea mays* L.) seeds were stored in canvas bags or in tin cans with screw tops in a cold room at approximately  $-5^{\circ}$  C. and in a controlled temperature chamber at  $30^{\circ}$  C. Full germination capacity and vigor was maintained for 9 years in the closed containers at  $-5^{\circ}$  C. Lots left open to the dry atmosphere of the  $30^{\circ}$  C. storage cabinet kept as well as those in open storage in a moisture-saturated atmosphere at  $-5^{\circ}$  C. for 7 years, though germination capacities were low. Moisture contents of the former were reduced to about 3 per cent of the dry weight of the seeds, while those of the latter were increased to 17 to 19 per cent. Storage of seeds with an initial moisture content of about 11 per cent in closed containers at  $30^{\circ}$  C. proved deleterious, resulting in reduced germination after 1 year.

## INTRODUCTION

Frost-damaged corn results from the exposure of immature grain to low temperatures. The extent of the damage depends upon the stage of maturity, the temperature reached, and the duration of exposure to the low temperature. Such corn is of inferior food value (5), and its value as seed is questionable.

An excellent description of freezing injury has been given by Kiesselbach and Ratcliff (4), and Rossman (7) has determined the extent of damage to maturing corn resulting from freezing under controlled conditions in the laboratory. Rossman (6, 8) has further investigated the freezing injury sustained by inbred and hybrid maize seed and found significant differences in freezing tolerance among inbred lines, though freezing tolerance was not manifested in hybrid vigor.

In spite of the considerable amount of work which has been done on the freezing injury of corn, no long-term experiment on the keeping quality of such seeds has been reported. Whitcomb (10) stated that the seed value of frosted wheat is uncertain without laboratory germination tests, and that such tests are most reliable when they are made six months or more after harvest.

## MATERIALS AND METHODS

Five lots of frost-damaged hybrid sweet corn (*Zea mays* L.) seeds were received in January 1951 from Dr. Clarke Q. Brown, Crookham Company, Caldwell, Idaho. They were as follows: Golden Jewel, Lot 7204; Golden Cross Bantam, Lot 7243; Golden Cross Bantam, Lot 7286; Golden Security, Lot 7336; and Stowell's Evergreen, Lot 7503.

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Injury information in regard to these seed lots was furnished by Dr. Brown (personal communication) and is shown in Table I.

Mechanical injury to corn seed during processing resulting in cracked or broken grains depends to a large extent upon the type of harvest. Combine harvesting causes most injury, mechanical harvesting causes medium injury, and hand harvest causes least injury. The injury to the seed as set forth in Table I refers to pericarp injury during the harvesting and shelling operations. Seeds with no pericarp injury apparent, even after staining, when examined under a low-powered reading glass are classified as free from injury. Degrees of injury to pericarp range from slight to heavy. The millout percentage is the amount of material discarded by the mills and machines during processing. This consists of undesirable kernels, too large,

TABLE I  
DATA OBTAINED ON FROST-DAMAGED SEED LOTS AT THE TIME OF RECEIPT AT THE  
PROCESSING PLANT. (FROM DR. CLARKE Q. BROWN)

| Lot No. | Type harvest | Amount (%) of pericarp injury |        |        |       | Moisture content, % | Millout, % | Final germination, % |
|---------|--------------|-------------------------------|--------|--------|-------|---------------------|------------|----------------------|
|         |              | Free                          | Slight | Medium | Heavy |                     |            |                      |
| 7204    | Combine      | 2                             | 65     | 0      | 33    | 16                  | 60         | 71                   |
| 7243    | Combine      | 70                            | 18     | 3      | 9     | 14                  | 20         | 80                   |
| 7286    | Combine      | 6                             | 81     | 8      | 5     | 11                  | 24         | 88                   |
| 7336    | Hand         | 9                             | 90     | 1      | 0     | 14                  | 48         | 90                   |
| 7503    | Mechanical   | 23                            | 68     | 4      | 5     | 27                  | 16         | 94                   |

too small, ill-shaped, or broken. The percentage of millout is determined as the seeds come from the field, and ordinarily runs about 15 per cent. Frost damage may increase millout to as much as 60 per cent as seen in Table I for Lot No. 7204. Heavier millout of frost-damaged seeds may raise the germination of marginal lots to make the seeds acceptable for planting. For example, for Lot No. 7204 the 40 per cent of the original harvest remaining after millout gave 71 per cent germination.

The moisture content shown in Table I was determined upon arrival of the seeds at the plant at Caldwell, Idaho.

Each lot of ten pounds was divided into four parts, which were stored in canvas bags or in tin cans with screw tops (not hermetically sealed) in a cold room at approximately  $-5^{\circ}$  C. and in a controlled temperature chamber at  $30^{\circ}$  C. The cold room had a saturated atmosphere while the relative humidity of the  $30^{\circ}$  C. chamber was very low.

Germination tests were made at the time of storage by placing replicates of four lots of 100 seeds each in moist, rolled paper towels at a daily alternating temperature of  $20^{\circ}$  to  $30^{\circ}$  C. At the same time the same number of seeds were used to determine the seedling-production capacity in soil in the greenhouse. The same methods of testing were used to determine

viability after 1, 3, and 6 months and 2.5, 3, 4, 6, 7, 8, and 9 years of storage. To conserve seeds, all viability tests made after storage consisted of duplicates of 50 seeds each.

Moisture content of all lots at the time of storage was about 11 per cent calculated on the basis of the dry weight of the seeds.

### RESULTS AND DISCUSSION

Data obtained from viability tests of three of the seed lots at various intervals are shown in Table II. Results from the other two lots were similar.

Germination on moist paper towels at a daily alternating temperature of 20° to 30° C. was, in general, higher than that in soil in the greenhouse. The latter is also a measure of the vigor of the seedlings since only those capable of emerging through the soil are counted as germinated.

It is evident that frost-damaged corn can be kept viable for at least 9

TABLE II

CHANGES IN VIABILITY AND MOISTURE CONTENT OF THREE LOTS OF FROST-DAMAGED CORN SEEDS STORED UNDER DIFFERENT CONDITIONS FOR NINE YEARS

| Sample of seed                                |                                    | Storage conditions |             | Percentage of germination after storage for years  |            |            |          |           |          |           | Per cent moisture after 9 years |  |
|-----------------------------------------------|------------------------------------|--------------------|-------------|----------------------------------------------------|------------|------------|----------|-----------|----------|-----------|---------------------------------|--|
| Lot No.                                       | Germination at start of storage, % | Temp., ° C.        | Container   | 0.5                                                | 1.0        | 2.5        | 3.0      | 6.0       | 8.0      | 9.0       |                                 |  |
|                                               |                                    |                    |             | Germination on moist filter paper at 20° to 30° C. |            |            |          |           |          |           |                                 |  |
| 7243                                          | 88                                 | -5                 | Open Sealed | 99<br>98                                           | 98<br>98   | 92<br>92   | 87<br>98 | 82<br>97  | 41<br>92 | 37<br>100 | 17.3<br>12.8                    |  |
|                                               |                                    | 30                 | Open Sealed | 97<br>96                                           | 97<br>75   | 89<br>5    | 90<br>0  | 60<br>—   | 11<br>—  | 4<br>—    | 3.0<br>5.7                      |  |
| 7286                                          | 97                                 | -5                 | Open Sealed | 99<br>98                                           | 99<br>99   | 94<br>95   | 98<br>98 | 54<br>100 | 43<br>94 | 33<br>98  | 19.1<br>12.4                    |  |
|                                               |                                    | 30                 | Open Sealed | 98<br>98                                           | 100<br>98  | 93<br>1    | 91<br>0  | 48<br>—   | 6<br>—   | 1<br>—    | 2.7<br>6.7                      |  |
| 7336                                          | 86                                 | -5                 | Open Sealed | 99<br>100                                          | 100<br>100 | 100<br>100 | 94<br>94 | 87<br>100 | 53<br>99 | 28<br>99  | 17.7<br>11.9                    |  |
|                                               |                                    | 30                 | Open Sealed | 100<br>100                                         | 99<br>100  | 99<br>39   | 98<br>0  | 94<br>—   | 44<br>—  | 35<br>—   | 3.0<br>5.3                      |  |
| Seedling production in soil in the greenhouse |                                    |                    |             |                                                    |            |            |          |           |          |           |                                 |  |
| 7243                                          | 78                                 | -5                 | Open Sealed | 83<br>79                                           | 79<br>80   | 84<br>79   | 80<br>90 | 51<br>86  | 21<br>80 | 10<br>82  |                                 |  |
|                                               |                                    | 30                 | Open Sealed | 83<br>70                                           | 81<br>54   | 74<br>0    | 78<br>0  | 31<br>—   | 3<br>—   | 0<br>—    |                                 |  |
| 7286                                          | 85                                 | -5                 | Open Sealed | 91<br>86                                           | 86<br>85   | 92<br>87   | 85<br>89 | 27<br>89  | 12<br>89 | 2<br>91   |                                 |  |
|                                               |                                    | 30                 | Open Sealed | 86<br>86                                           | 84<br>67   | 80<br>0    | 76<br>0  | 19<br>—   | 0<br>—   | 0<br>—    |                                 |  |
| 7336                                          | 79                                 | -5                 | Open Sealed | 86<br>91                                           | 77<br>81   | 81<br>83   | 84<br>83 | 65<br>83  | 46<br>88 | 15<br>81  |                                 |  |
|                                               |                                    | 30                 | Open Sealed | 82<br>88                                           | 82<br>74   | 78<br>18   | 81<br>0  | 67<br>—   | 22<br>—  | 0<br>—    |                                 |  |



years if it is stored in a closed tin can at approximately  $-5^{\circ}$  C. This means, then, that its seeding value remains unimpaired for this period of time in spite of the original injury. Also all lots held at  $30^{\circ}$  C. exposed to a dry atmosphere germinated as well after 7 years as those stored in a saturated atmosphere at  $-5^{\circ}$  C. though all lots showed decreased viability.

This points once again to the important interrelation of temperature and moisture for the retention of seed viability. The moisture content of the seeds after storage for 9 years under the various conditions is shown in Table II. It is apparent that seeds held in a saturated atmosphere at  $-5^{\circ}$  C. were much more moist than those at  $30^{\circ}$  C. Seeds in closed containers at  $-5^{\circ}$  C. changed very little in moisture during storage, though a small increase was noted; i.e., from about 11 per cent at the beginning to 11.9 to 12.8 per cent after 9 years. In other words, a small amount of moisture was absorbed by the seeds due to the fact that they were not hermetically sealed, but were covered with a screw cap. Moisture content of seeds stored in open containers under the same conditions increased up to 19.1 per cent moisture.

At  $30^{\circ}$  C., on the other hand, open storage in the very dry atmosphere reduced the moisture content to about 3 per cent of the dry weight of the seeds for all lots. This reduction permitted the retention of full viability for three years; the same length of time seeds remained capable of vigorous germination after being held at  $-5^{\circ}$  C. with moisture contents of 17 to 19 per cent. The similar effects of the open storage at  $30^{\circ}$  C. and  $-5^{\circ}$  C. are shown graphically in Figure 1, for Lot No. 7503.

In view of the fact that the moisture content in closed containers in the  $30^{\circ}$  C. chamber was reduced to 5.3 to 6.7 per cent after 9 years of storage, and since this is a safe moisture content for storage, it might be expected that these seeds would also have an extended life span. Actually, however, deterioration was very rapid under these conditions (Table II and Fig. 1). The explanation for this is to be found in the fact that the seeds were adversely affected immediately by the high initial moisture content (11 per cent) and the high temperature. This early damage led to rapid deterioration and early death of the seeds. This same effect has been shown for several other seeds, namely dandelion, onion, eggplant, and tomato (1, 2), where the initial moisture content has been unfavorably high and has fluctuated downward with storage.

All of these frost-damaged corn seeds were kept successfully for 9 years in closed containers at  $-5^{\circ}$  C. Not only did they retain their germination capacity as indicated by tests on moist paper towels at controlled temperature, but their vigor also remained apparently unimpaired, as shown by the production of seedlings in soil in the greenhouse. This is shown in Figure 2. Here, it is seen that seedling production in soil after 8 years of

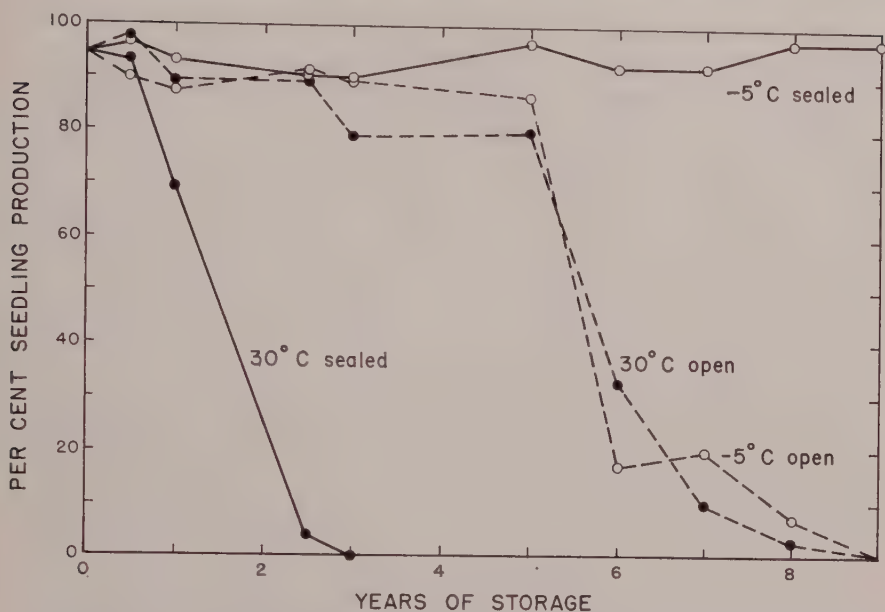


FIGURE 1. Seedling production in soil in the greenhouse from frost-damaged corn (Stowell's Evergreen, Lot 7503) after storage for various lengths of time in open or closed containers at  $-5^{\circ}$  or at  $30^{\circ}$  C.

storage in open containers at  $30^{\circ}$  C. (A) was not only reduced but was delayed as compared with storage in open containers at  $-5^{\circ}$  C. (B). Seeds in closed containers at  $-5^{\circ}$  C. remained capable of full, vigorous germination and growth (Fig. 2 C).

It can be concluded, then, from these tests, that frost-damaged corn seeds can be used for planting up to 9 years after harvest, provided they

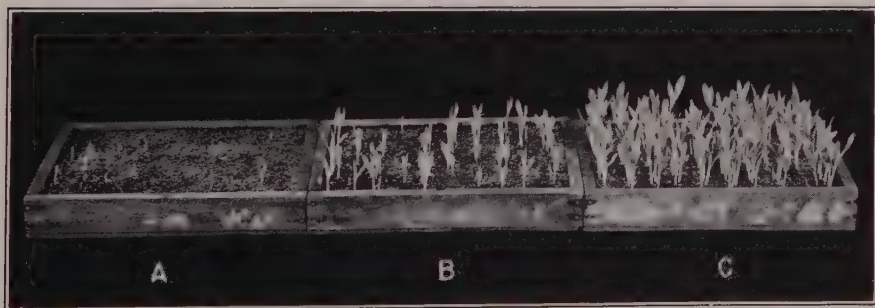


FIGURE 2. Seedling production in 11 days from frost-damaged corn (Golden Security, Lot 7336) stored for 8 years in open containers at  $30^{\circ}$  C. (A) or  $-5^{\circ}$  C. (B), or in a closed container at  $-5^{\circ}$  C. (C).

are stored in closed containers at  $-5^{\circ}\text{C}$ . Other storage conditions would hold them safely for shorter lengths of time. In fact there is no reason to believe that frost-damaged corn seeds cannot be held as long as non-damaged seeds and still retain their full original germination capacity provided they are given proper storage conditions. Unpublished data on high quality corn stored at this Institute indicate a similar deterioration rate under the same conditions. Similar results for nondamaged seeds have been obtained by Boswell *et al.* (3) and by Sayre (9) who stored corn with various moisture contents at several different temperatures.

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# PRODUCTION OF PEACH SEEDLINGS FROM UNCHILLED SEEDS. I. EFFECT OF NUTRIENTS IN THE ABSENCE OF COTYLEDONARY TISSUE

FLORENCE FLEMION AND PEGGY LUSSES PROBER

## SUMMARY

A method is described whereby apparently normal seedlings can be obtained from unchilled seeds of a variety of peach [*Prunus persica* (L.) Stokes] which require approximately nine weeks of 5° C. for after-ripening. The excised embryos, from which the cotyledons have been removed, are grown under aseptic conditions on nutrient agar at room temperature. Within 10 to 20 days, after adequate root development has taken place, they are transferred to nutrient culture in the greenhouse where they subsequently produce plants which appear to be normal. Whether the development of the seedlings under these conditions is due to the removal of inhibiting substances which may be present in the cotyledons or to the fact that axillary shoots develop and assume dominance has not been definitely determined. The evidence favors the latter viewpoint.

Although germination of the ecotyledonized embryos from both chilled and unchilled seeds occurred on the nutrient agar, it was slower and more erratic in the latter. In attempts to overcome this condition the following substances were added to the agar cultures: coconut milk, yeast extract, adenine sulfate, casein hydrolyzate, diphenylurea, indole-3-acetic acid, 1-naphthaleneacetic acid, kinetin, fructose-1,6-diphosphate, glucose-1-phosphate, extracts of chilled and unchilled cotyledons as well as of ecotyledonized embryos. None of these in the concentrations tested had any apparent effect in accelerating the development of either the roots or shoots; and likewise the addition of extracts from unchilled seeds did not produce physiological dwarfs. Such abnormal seedlings were obtained, however, when unchilled seeds with cotyledons attached were germinated on nutrient agar, then transferred, either with or without cotyledons, to the liquid culture in the greenhouse.

## INTRODUCTION

Vigorous normal seedlings of peach [*Prunus persica* (L.) Stokes] are obtained by chilling the seeds while in a moist medium prior to germination, and depending upon the variety from 8 to 14 weeks at 5° C. are required (2, 6, 24). Plants produced by removing the seed coats and forcing the germination of unchilled seeds are physiologically dwarfed; that is, the short telescoped stems terminate in a rosette of leaves which are frequently abnormal (3, 4, 5, 17, 19, 20, 21, 22, 24).

When the cotyledons of either chilled or unchilled seeds are removed, the ecotyledonized embryos must be cultured on nutrient agar in order to obtain germination (7, 14). The shoot growth under these conditions consists of a slight elongation of the first three to five internodes with a cluster of embryonic leaves at the tip. When transferred to liquid cultures in the

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greenhouse, after 10 to 21 days on the nutrient agar, apparently normal seedlings are obtained. These results have been published previously in abstract form (7).

#### MATERIALS AND METHODS

Peach seeds from a propagated selected wild strain, known as "Superior Peach Pits," grown in Tennessee were obtained in 1956 shortly after harvest, and stored dry in closed containers at 5° C.

Upon removal of the hard outer coat (pericarp) with the aid of a cracking device (6), the dry unchilled seeds with inner coats intact were surface sterilized by submersion for 5 minutes in a 5 per cent solution of calcium hypochlorite (28). They were then taken through several rinses in sterile tap water under aseptic conditions and, upon soaking overnight (sterile tap water in a stoppered container) at room temperature, the seeds were aseptically excised and placed on sterile agar slants. Usually both cotyledons were removed (ecotyledonized); however, in several experiments one-third to one-half of one cotyledon was retained. The nutrient medium (pH 5.6) consisted of 0.7 per cent Difco Bacto-agar, 2 per cent sucrose, major and minor elements in p.p.m. as recommended by Heller (11, p. 199), biotin, calcium pantothenate, cysteine-hydrochloride, inositol, and thiamine as suggested by White (27, pp. 75-76).

Various substances were added to the basic nutrient and these are indicated in the text. One of these additions was extracts of cotyledons from chilled and unchilled seeds. The cotyledons (100 or more) were homogenized and extracted (1.5 ml. of solvent for each cotyledon) with acetone, then 80 per cent ethanol, and finally with distilled water. After each extraction the residue obtained upon filtering was placed in the next solvent while the filtrate was evaporated to dryness and resuspended in water. These various extracts were added, prior to sterilization (15 lb. pressure for 20 minutes), in such a manner that each 10 ml. agar slant contained the equivalent of one-fourth, one-tenth, or one-fortieth of one cotyledon. Ecotyledonized embryos from chilled seeds were also extracted, but only with water, and with this material each agar slant contained the equivalent of one embryo.

The agar cultures were held under various conditions as follows: in a constant light room (8, p. 58), in the laboratory with an eastern exposure to sunlight, and in the greenhouse with or without supplementary light (8, pp. 58-59). Under these conditions some of the embryos developed sufficient roots, usually within 10 days to several weeks, so that they could be transferred; this was done in the late afternoon on a cool cloudy day. Rectangular polyethylene pans (9-liter capacity), with covers, were completely covered (sides, top, and bottom) with aluminum foil in order to exclude light. At the time of transfer an opening was made in the foil over

one of the holes (each 12 mm. diameter) in the cover and the tiny seedling was carefully placed in position with the root submerged in the nutrient solution and the epicotyledonary axis exposed to light. Each seedling was firmly anchored in position with nonabsorbent cotton which was placed in the hole around the stem. Within 3 to 5 days after transfer to the liquid culture aeration was started and maintained thereafter through a tube inserted in one of the holes in the cover. Approximately 12 seedlings were grown in each pan.

The solution of macronutrient salts and micronutrient elements was similar to that described by Weinstein and Robbins (26, p. 226) except that iron in 1 p.p.m. was supplied in a chelated form. All of the liquid cultures were maintained in the greenhouse and in a number of experiments daylight was supplemented with light at night (8, pp. 58-59). The pH of the liquid nutrient was taken at regular intervals and as the seedlings developed luxuriant top growth and large fibrous roots, a shift in pH from 5.2 to above 6.0 would occur within a relatively short period of time. Thus, at this stage of development the nutrient solution was changed every second or third day, whereas at the beginning of the experiment when growth was slow it was renewed weekly. Seedlings produced in this manner were compared with normal seedlings obtained from seeds which had been adequately chilled prior to germination (2, 6).

For microscopical examination of the apical tissue the material was fixed in formalin-aceto-alcohol (13, p. 41), dehydrated in ethanol, paraffin (Fischer's tissue-mat 54°-56° C.) infiltrated and embedded, sectioned longitudinally at 10  $\mu$ , and stained with safranin O, then counterstained with Fast Green (13, p. 81).

## RESULTS

Peach embryos from unchilled seeds when germinated on nutrient agar produced roots as shown in Figure 1. When none of the cotyledonary tissue had been retained (Fig. 1 C), it was essential in order to obtain germination to culture the ecotyledonized embryos of both chilled and unchilled seeds in this manner; however, the development of the roots especially those from unchilled seeds was slow and erratic. Usually, by the second or third week the shoot growth of ecotyledonized embryos (6 to 10 mm.) consisted of a slight elongation of the first 3 to 5 internodes which later terminated in a rosette of stipules and immature leaflets (Fig. 1 C). On the other hand, when part of the cotyledons had been retained, growth of the embryos from unchilled seeds occurred equally well whether on the nutrient agar (Fig. 1 A, B) or on moist filter paper in Petri dishes at room temperature. Germination and subsequent development were both vigorous and rapid so that within ten days on the agar, especially when greater amounts of the cotyledons had been retained, the tips of the shoots sometimes reached

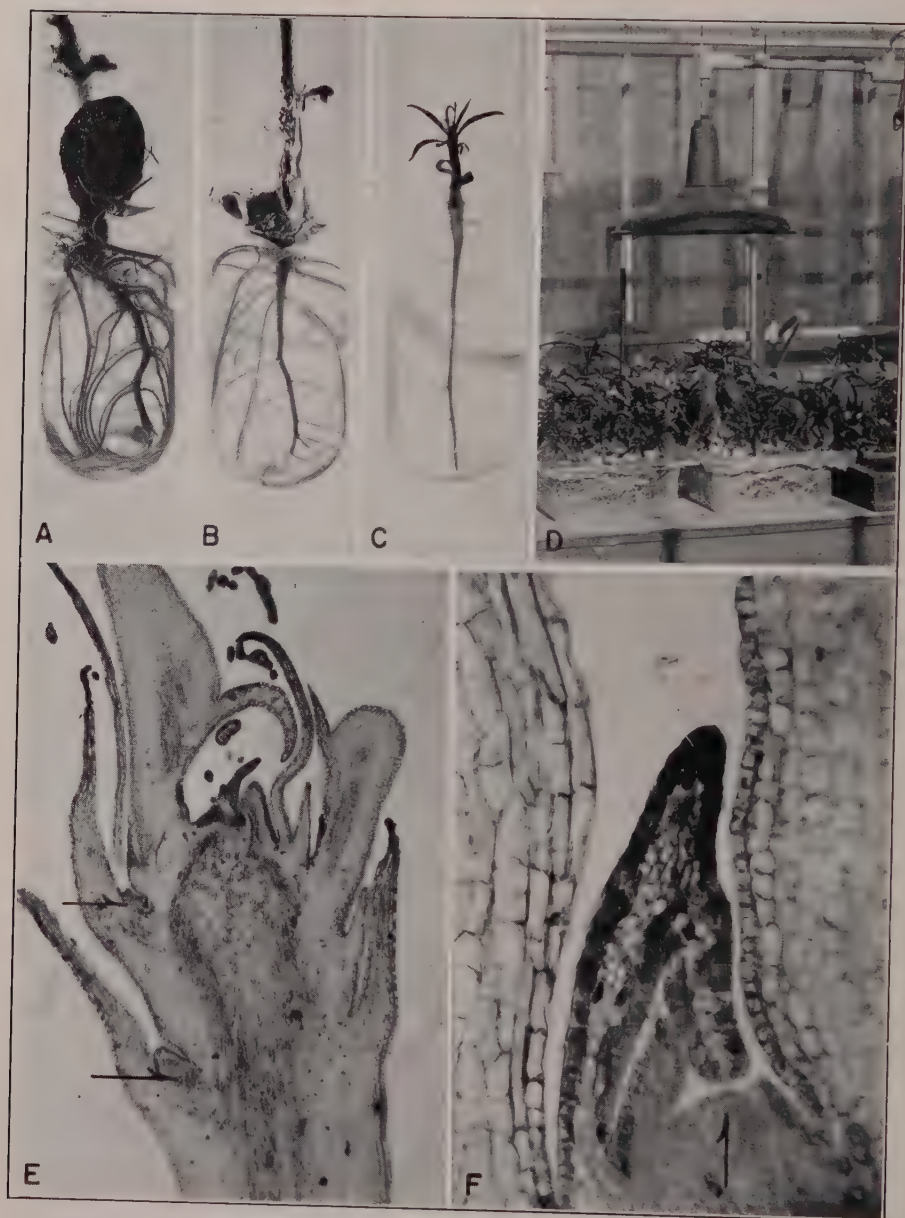


FIGURE 1. Development of excised embryos from unchilled peach seeds with (A) both cotyledons, (B) one-half of one cotyledon, and (C) no cotyledons on nutrient agar at room temperature after 16 (A and B) and 29 (C) days. ( $\times 0.9$ ) (D) Seedlings from ecotyledonized embryos growing in nutrient solution in the greenhouse with supplementary light at night. (E and F) Sectioned apical region of shoot similar to C. Arrows indicate axillary buds. (E,  $\times 36$ ; F,  $\times 225$ )



the cotton plugs at the top of the tubes. Adequately chilled seeds with part of the cotyledons retained germinated so rapidly within a few days in the Petri dishes that agar culture was not necessary.

To obtain seedlings from ecotyledonized embryos which had germinated on nutrient agar it was necessary at the stage shown in Figure 1 C, to transfer to liquid nutrient. Unchilled seeds with part of one cotyledon retained which had germinated on either nutrient agar (Fig. 1 B) or on moist filter paper were also subsequently grown in nutrient solution, but when transferred to the liquid culture the cotyledonary tissue which had been present during germination was removed in some cases while in others it was retained for the duration of the experiment. Upon transfer to the liquid culture, there followed a period during which there was no visible shoot growth; however, during this interval secondary roots were gradually developing. This so-called quiescent period was much longer (sometimes up to 10 days) for the seedlings from ecotyledonized embryos than for those in which some of the cotyledonary tissue had been retained during the period on nutrient agar.

When ecotyledonized embryos of seeds (whether chilled or unchilled) were cultured on nutrient agar, then subsequently grown in liquid solution, the seedlings obtained appeared to be normal (Fig. 2 B). Of the 81 plants from unchilled seeds produced in this manner the stems and leaves resembled those of normal plants (Fig. 3 E) except for one seedling which produced two tiny, curled leaves about half-way up the stem. On the other hand, seedlings from unchilled seeds with all or only part of the cotyledons present during the initial growth period (germination on either nutrient agar or moist filter paper at room temperature) and subsequently held in liquid nutrient with either none (Fig. 2 A) or some (Fig. 2 C) of the cotyledonary tissue retained had the characteristics of physiological dwarfs, that is, short internodes, curled leaves on the epicotyledonary axis, and in addition produced many lateral shoots (Fig. 2 A, C). These plants were similar in appearance to those illustrated in Figure 3 B, C, D. The differences in seedlings shown in Figure 2 A and C, as compared with Figure 2 B, persisted whether grown in the greenhouse with or without supplementary light at night. However, with additional light (Fig. 1 D) the main axis was more elongated while the other features were unchanged.

In other greenhouse experiments seedlings were grown in sand culture instead of in the aerated nutrient solution. Both chilled and unchilled seeds with cotyledons intact were germinated on moist filter paper, then transferred to clay pots containing sand kept moist with nutrient solution added three times weekly with supplementary watering to the clay saucers which held the pots. These cultural conditions produced a profusion of lateral shots as seen in Figure 3 in both the physiologically dwarfed





FIGURE 2. Seedlings from unchilled peach seeds after 29 (A), 39 (B), and 47 (C) days in nutrient solution in the greenhouse. Cotyledons removed immediately following germination (A) and prior to germination (B). (C) Cotyledons intact throughout entire experiment. Note short telescoped stems with abnormal leaves on main stems in A and C and profusion of lateral shoots in C. Subsequently seedlings in A also produced lateral shoots while those in B continued to develop as shown. ( $\times 0.14$ )

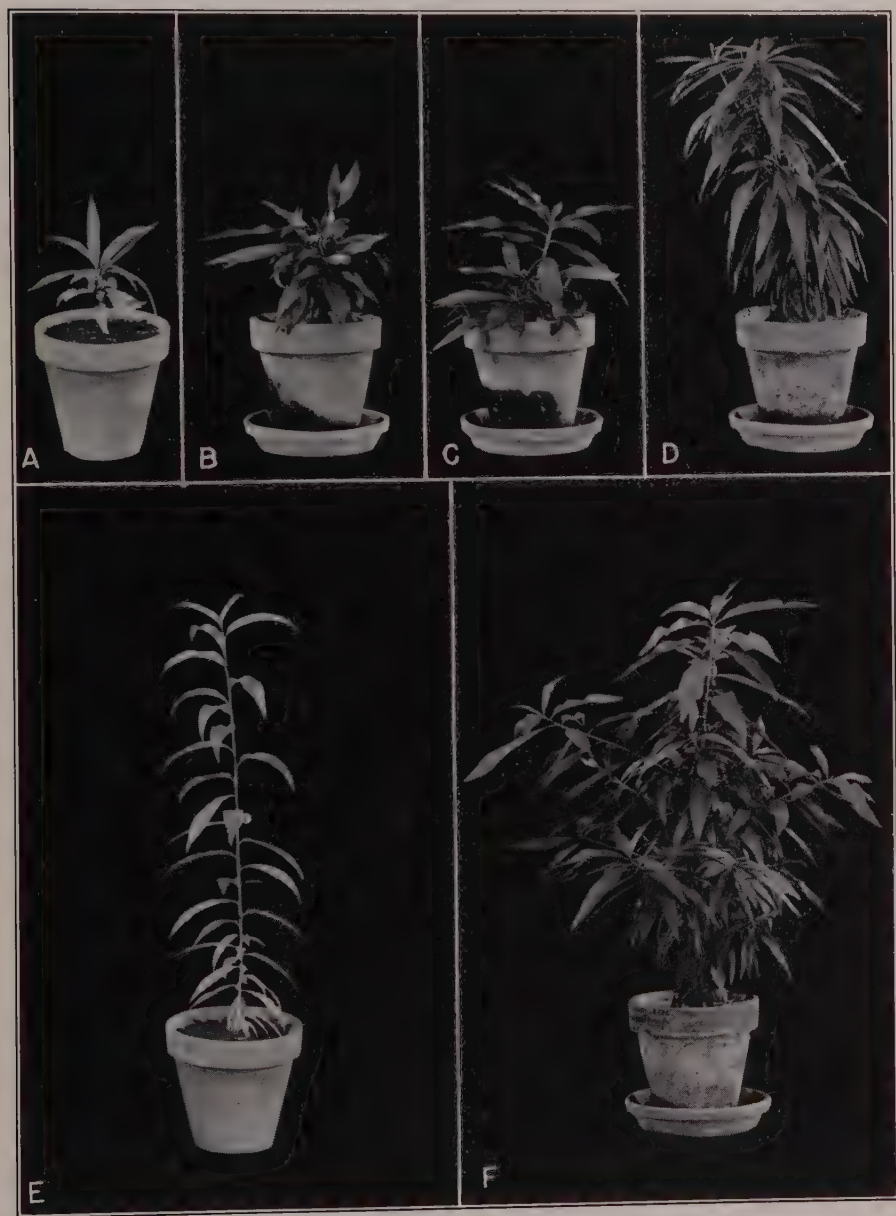


FIGURE 3. Development of seedlings in the greenhouse from unchilled (A-D) and adequately chilled (E, F) seeds of peach. (B, C, D, and F) Grown in nutrient sand culture (note production of laterals) and (A, E) in soil. ( $\times 0.12$ )

(Fig. 3 B, C, and D) and normal (Fig. 3 F) seedlings. Typical plants produced when grown in soil are illustrated in Figure 3 A and E.

Since, as shown above, axillary buds readily produced shoots, the question arose as to whether the so-called normal seedlings obtained from ecotyledonized embryos were due to lateral shoots which assumed ascendancy, for in such a case the stems and leaves would be normal. It was not possible by visual inspection of the tiny compressed apex to locate the origin of new growth; therefore, a microscopical examination of the apical region was undertaken at the time the plantlets were transferred from the nutrient agar to the liquid culture. This revealed that axillary buds (Fig. 1 E) were not only prevalent but in some cases had progressed to the stage of having a number of well-developed leaf primordia (Fig. 1 F). Since only one shoot was produced on each seedling it is possible that one of these lateral buds assumed dominance during the so-called quiescent period in the liquid nutrient just prior to resumption of top growth.

Attempts were made to encourage a greater and more rapid development of both the root and the epicotyledonary axis of ecotyledonized embryos while on the nutrient agar. Such an initial stimulus might produce a more vigorous and sturdier plantlet and upon transfer to the liquid solution might reduce or eliminate the lag period for subsequent resumption of growth. This type of effect was not obtained by the addition to the basic nutrient agar of any of the following substances: coconut milk (20, 10, 6, 5, 4, 3, and 2 per cent), yeast extract (0.05 per cent), adenine sulfate (10, 0.25, 0.1, and 0.05 mg./l.), casein hydrolyzate (200, 20, 2, and 0.2 mg./l.), diphenylurea (15, 3, 0.6, and 0.12 mg./l.), indole-3-acetic acid (10, 2, 0.4, 0.08, and 0.016 mg./l.), 1-naphthaleneacetic acid (0.3 and 0.1 mg./l.), kinetin (1, 0.2, 0.04, and 0.008 mg./l.), fructose-1,6-diphosphate (1 per cent), the potassium salt of glucose-1-phosphate (1 per cent), water extracts of chilled ecotyledonized embryos and acetone, 80 per cent ethanol, and water extracts of both chilled and unchilled cotyledons. While many of these materials induced callus formation and thus prevented germination, none of the concentrations tested had any stimulatory effects on the development of either the root or shoot of the ecotyledonized embryos.

#### DISCUSSION

A number of investigators have been interested in the embryo culture of peach, but their primary concern has been the possibility of obtaining seedlings from crosses of early-ripening varieties. In addition, various techniques were developed for early-, mid-, and late-maturing varieties (1, 4, 10, 12, 15, 16, 18, 23, 25) and the plants obtained were either physiologically dwarfed or normal, depending upon whether or not the chilling



requirements of the seeds had been adequately satisfied. In regard to the culture of embryos in the absence of any cotyledonary tissue, Kester (14) obtained germination of such ecotyledonized embryos from both chilled and unchilled seeds, but the initial development (within 10 to 14 days) could not be improved by transference to a fresh agar medium, whereas, in the present studies, seedlings were obtained when subsequently cultured in liquid nutrient in the greenhouse.

The chilled cotyledonary tissue when present during germination exerts a tremendous influence on seedling development since normal plants are produced even when only a small amount is retained—this latter obtains miniatures which are, however, normal (9, 14). Likewise, the unchilled cotyledons also have a far-reaching effect on subsequent development for when present even in small amounts during germination (see text above) the elongation of the epicotyledonary axis is inhibited and, in addition, abnormal leaves are frequently produced. Since the seedlings from ecotyledonized embryos of both chilled and unchilled seeds (when cultured as described in the text above) appeared to be normal, one immediately conjectures that the materials essential for normal development of the epicotyledonary axis and leaves were either lacking or unavailable in the unchilled cotyledons or that inhibiting substances were present.

There are, however, other possibilities such as an axillary bud assuming dominance. It is well-known that physiologically dwarfed seedlings during their initial growth tend to produce lateral shoots (1, 4, 8, 18, 20, 24, 25) which have normal internodes; these laterals do not in turn produce laterals, and except for a few rare instances have normal leaves. It has been shown (24, p. 439) that when one of these shoots assumes ascendancy it gives the misleading appearance of normal development of the main axis. In regard to the origin of the shoot in seedlings from ecotyledonized unchilled embryos, a microscopical examination at the time of transfer from the agar to the liquid culture revealed that at the base of some of the small leaflets there were axillary buds, some of which had well-developed leaf primordia. It is quite conceivable that if such a lateral bud so near to the tiny compressed tip produced a shoot, it could easily assume ascendancy. In such an event the seedling would appear normal in every respect and would not, in turn, produce lateral shoots.

Of the 81 seedlings obtained from the ecotyledonized embryos of unchilled seeds, all had normal stems, internodes, and leaves except for one plant which had two tiny abnormal leaves. This latter could possibly be due to the influence of a very small amount of cotyledonary tissue which may have been present or to a rare occurrence in physiological dwarfs whereby several curled leaves appear on an otherwise normal lateral shoot (8, p. 61; 24, p. 439). In numerous experiments in this laboratory with



peach in the past, this usual condition occurred on shoots produced at or very near a telescoped apex which appeared either damaged or dead. In any event abnormal leaves are never found on either the main axis or lateral shoots of normal seedlings obtained from adequately chilled seeds.

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### Note

## THE IMPORTANCE OF WETTING AGENTS AS AFFECTING THE TOXICITY OF CERTAIN INSECTICIDES

ALBERT HARTZELL AND FRANK WILCOXON

Conflicting reports<sup>1</sup> from other laboratories as to the toxicity of *S*-(2,3-dicarbomethoxypropyl)-*O,O*-dimethyl phosphorodithioate induced the writers to conduct the following tests to determine why such differences should occur. This compound (165-1591 A) which was used by the writers in the present tests was received from Chas. Pfizer & Co., Inc. An initial test using the methods described below showed that the above-named compound possessed marked toxicity at 40 p.p.m. to the two-spotted mite, *Tetranychus telarius* (L.), which is in agreement with results previously obtained.

Parallel tests run by an independent group at Boyce Thompson Institute, also using the methods described below, showed that when the wetting agent Triton X-155, an alkyl aryl polyether alcohol, was used alone at the rate of 2 and 4 p.p.m. of spray solution the results did not differ significantly from the untreated check. When, however, the same wetting agent was used at 5000 p.p.m. of diluted spray, marked toxicity to the two-spotted mite resulted. The mites used by the two groups of investigators were from the same colony.

### MATERIAL AND METHODS

The testing methods and holding of the plants after spraying were identical, except for all formulations. Both adult and young mites reared on *Phaseolus vulgaris* L. var. Tendergreen beans were used in the tests. One day before spraying, infested leaves were cut from the stock culture and placed on the leaves of new plants in 2½ in. pots, each pot containing two plants. Enough infested leaf area was used to insure a population of approximately 50 mites on each test plant leaf.

*Formulation.* The following procedure was used in formulating test chemicals for spray tests: 100 mg. of the active ingredients of the toxicant was placed in 100 ml. of acetone, which is equivalent to a 1000 p.p.m. solution. For 20 p.p.m., 2 ml. of the stock solution, 2 ml. of acetone, and the proper amount of wetting agent were combined with water to give a total volume of 100 ml. The 40 p.p.m. solution differed only in that 4 ml. of the stock solution were included in the 100 ml., and it was unnecessary to add any acetone since both now contained 4 ml. of acetone.

<sup>1</sup> Letter from Dr. George L. McNew to Mr. Richard A. Benedict, Chas. Pfizer & Co., Inc., Brooklyn, New York.



The test compounds were formulated by solution or suspension in water and the addition of the wetting agent. Malathion which was used as a standard and the test compound were run at two concentrations. The wetting agent used was Triton X-155, an alkyl aryl polyether alcohol, obtained from Rohm & Haas Co., Philadelphia.

*Application of toxicant.* A turntable spray-hood and a DeVilbiss spray gun were used for applying the test compounds (2). The turntable rotated at 18 r.p.m. and the plants were sprayed for 6 full revolutions. The output of the gun during this application, at a pressure of 40 lb. per sq. in. and a siphon height of 6 in. was approximately 80 ml. The distance of the nozzle to the center of the turntable was 54 inches.

*Holding conditions.* The test plants were held for 12 hr. under light in a room at 79° to 82° F., and a relative humidity of 40 to 50 per cent until counts were made.

*Recording results.* All mites that exhibited movement when examined under a binocular microscope were considered alive; those showing no movement were considered dead. Counts for immediate acaricidal effect were made at the end of 48 hr. At each observation, two separate samples were taken from the test leaves. The results were averaged to give the percentage mortality.

## RESULTS<sup>2</sup>

Toxicity tests were run at two concentrations of *S*-(2,3-dicarbomethoxypropyl)-*O,O*-dimethyl phosphorodithioate, namely 20 and 40 p.p.m., alone and with three different concentrations of the wetting agent, namely 2500, 5000, and 10,000 p.p.m. as without a wetting agent. The results are shown in Table I. Each percentage kill given is an average of three determinations. Each value shows the effect on mortality of increasing concentrations of toxicant at a fixed concentration of wetting agent.

It will be seen that as the wetting agent concentration was increased the general level of toxicity increased. Without the wetting agent, the average percentage kill obtained even at the highest concentration of this compound (Table I, Toxicant I) did not exceed 11 per cent (kill), whereas with the highest level of wetting agent the average percentage kill was about 88 per cent. This clearly shows that sufficient amounts of wetting agents are necessary in order for the toxicity of the compound to be exhibited and experiments with minute amounts of wetting agents may be expected to give very low toxicity values.

In the case of malathion, *S*-(1,2-dicarboethoxyethyl)-*O,O*-dimethyl phosphorodithioate (Table I, Toxicant II), there also was found a similar effect of increased insecticide toxicity with increased concentration of wetting agent with one exception.

<sup>2</sup> The writers are indebted to Mr. Robert Feinstein for technical assistance.

TABLE I  
RESULTS OF TESTS OF TWO ORGANIC PHOSPHATE INSECTICIDES IN  
COMBINATION WITH A WETTING AGENT\*

| Toxicant I**            |               |        |
|-------------------------|---------------|--------|
| Concentration in p.p.m. |               | % Kill |
| Toxicant                | Wetting agent |        |
| 0                       | 0             | 9      |
| 20                      | 0             | 10     |
| 40                      | 0             | 11     |
| 0                       | 2,500         | 9      |
| 20                      | 2,500         | 19     |
| 40                      | 2,500         | 71     |
| 0                       | 5,000         | 15     |
| 20                      | 5,000         | 78     |
| 40                      | 5,000         | 81     |
| 0                       | 10,000        | 23     |
| 20                      | 10,000        | 87     |
| 40                      | 10,000        | 88     |
| Toxicant II***          |               |        |
| 0                       | 0             | 5      |
| 20                      | 0             | 13     |
| 40                      | 0             | 31     |
| 0                       | 2,500         | 16     |
| 20                      | 2,500         | 25     |
| 40                      | 2,500         | 47     |
| 0                       | 5,000         | 29     |
| 20                      | 5,000         | 62     |
| 40                      | 5,000         | 62     |
| 0                       | 10,000        | 30     |
| 20                      | 10,000        | 54     |
| 40                      | 10,000        | 50     |

\* Triton X-155, an alkyl aryl polyether alcohol from Rohm & Haas Co., Philadelphia, Pa.

\*\* *S*-(2,3-dicarbomethoxypropyl)-*O,O*-dimethyl phosphorodithioate.

\*\*\* *S*-(1,2-dicarboethoxyethyl)-*O,O*-dimethyl phosphorodithioate (malathion).

Furmidge (1) has recently called attention to the fact that the importance of surface forces in agricultural sprays is often overlooked and cites the early work of Wilcoxon and Hartzell (4) in using wetting agents to increase the efficiency of insecticides. Wilcoxon and Morgan (5) also stressed the importance of surface active agents in foliage sprays. Similar effects have been shown by Turner, Saunders, and Willaman (3) in a recent bulletin on some polyethyleneglycol derivatives on the toxicity of nicotine to insects.

## SUMMARY

The kill obtained with a toxicant may vary from almost nothing to a substantial figure depending upon the concentration of the wetting agent employed and perhaps upon the wetting agent itself. For example, the addition of Triton X-155 at 10,000 p.p.m. to *S*-(2,3-dicarbomethoxypropyl)-*O,O*-dimethyl phosphorodithioate at 40 p.p.m. increased the kill of mites from 11 per cent to 88 per cent.

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# THE RANGE OF FREE AMINO ACIDS AND AMIDES IN TOMATO PLANTS AND THE EFFECTS OF NITRATE OR AMMONIUM AS NUTRIENTS<sup>1</sup>

DAVID MARGOLIS<sup>2</sup>

## SUMMARY

The experiments described relate to the effects of inorganic nitrogen nutrition upon the amino acids and amides soluble in 80 per cent ethanol in the leaves and roots of tomato plants. It has been confirmed that ammonium nutrition is correlated with a high amide level and nitrate nutrition with high levels of dicarboxylic acids. Tomato plants which received a complete nutrient medium but which were grown at different times responded to uncontrolled fluctuations in the environment with an altered nitrogenous composition. This paper shows the range of these effects and so presents a foundation for further reports of the effects due to manganese and molybdenum upon the free amino acids and amides of tomato plants.

## INTRODUCTION

This paper reports the first part of a study of the effects of manganese and molybdenum upon the ethanol-soluble amino acids and amides of the tomato (*Lycopersicon esculentum* Mill.) (8). This section will deal with the experimental procedures and with the composition of the experimental plants as they were grown in full nutrient solution with nitrogen supplied as nitrate or ammonium. The remaining parts will deal, in turn, with the effects of manganese and molybdenum and with a consideration of the functions of these trace elements in the metabolism of plants.

In order to evaluate the influence of the trace elements, it is necessary first to establish the nature of the amino compounds present in plants exposed to supposedly normal conditions of nutrition (i.e., a complete complement of nutrients) and also the fluctuations which may occur under the stress of the environmental conditions normally encountered during growth in a greenhouse.

An important feature of the later experiments lies in the manipulation of the plants to produce a maximum effect of the trace element and of the type of nitrogen supplied upon the soluble amino compounds. Usually, this was accomplished by withholding nitrogen from young plants grown for a period of time after the development of visible evidence of trace element deficiency. In this manner plants deficient in the trace element and low in nitrogen were obtained. Subsequently both the trace element in question and nitrogen were restored and during the recovery period sam-

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ples were obtained at intervals to establish the presence of unique effects that could be attributed to the influence of the trace element. Nitrate and ammonium nitrogen were supplied separately in the expectation that their individual effects would produce characteristic patterns of amino acids which would also be influenced by the trace element.

Young seedlings were used to provide actively growing plants and to obtain responsive material in the earlier stages of trace element deficiency. In this manner, plants in the complete nutrient solutions had high rates of metabolic turnover and deficiency symptoms could be established without encountering terminal effects as often seen in older plants.

#### MATERIALS AND METHODS

The following types of nutrient solutions were used in the course of the experimental work. All concentrations are expressed below in equivalents per liter of nutrient solution.

Complete nutrient solution with nitrogen supplied as nitrate: calcium nitrate 0.002, magnesium sulfate 0.002, potassium sulfate 0.002, potassium dihydrogen phosphate 0.001.

Complete nutrient solution with nitrogen supplied as ammonium: ammonium sulfate 0.001, magnesium sulfate 0.002, calcium chloride 0.002, potassium dihydrogen phosphate 0.002.

Nutrient solution lacking nitrogen: calcium chloride 0.003, magnesium sulfate 0.002, potassium sulfate 0.004, potassium dihydrogen phosphate 0.001.

High nitrogen solutions containing nitrate-N as applied to nitrogen deficient plants: calcium nitrate 0.0032, potassium nitrate 0.0037, magnesium sulfate 0.002, potassium sulfate 0.002, potassium dihydrogen phosphate 0.001.

High nitrogen solutions containing ammonium-N as applied to nitrogen deficient plants: ammonium sulfate 0.005, magnesium sulfate 0.002, potassium sulfate 0.002, potassium dihydrogen phosphate 0.001, calcium chloride 0.002.

The following trace elements were applied as required in the nutrient solutions at the concentrations named: iron (1.0 p.p.m.), boron (0.05 p.p.m.), zinc (0.05 p.p.m.), copper (0.02 p.p.m.), manganese (0.5 p.p.m.), and molybdenum (0.02 p.p.m.).

The major nutrient salts were purified with respect to manganese by either the alkaline earth-phosphate adsorption method (1) or by co-precipitation with iron and 8-hydroxyquinoline (3). Molybdenum was reduced to a minimum in the major nutrient solutions by the copper sulfide co-precipitation technique (10). Water low in trace element content was obtained by percolating steam distilled water through a column of strong anion- and cation-exchange resins [Amberlite 1RA-400 (OH<sup>-</sup>) and Amberlite 1R-120 (H<sup>+</sup>)].

The seedlings were grown either in Pyrex glass or polyethylene con-

tainers and were supported in lids of perforated polyethylene sheets with plastic wool used to maintain them in an erect position. The containers were covered with aluminum foil to exclude light. All containers were cleaned with a hot detergent solution, 10 per cent HCl, and finally rinsed with water prepared by ion-exchange techniques. Water was replenished daily and the nutrient solutions were renewed whenever the pH fluctuated by one unit. Aeration of the cultures was provided by a stream of air passed through glass wool and distilled water.

The tomato plants used in these studies were the Rutgers variety, Harris Special Strain. After sterilization with calcium hypochlorite, the seeds were germinated in acid washed sand which had been equilibrated with nutrient solution which was free of trace elements. Five days after germination the seedlings were transferred to nutrient solution, and the cotyledons were removed to limit the transport of inorganic nutrients to the growing regions from the storage organs. Continuous aeration was provided after several days.

When sampled, the plant material was killed and extracted in a VirTis 45 homogenizer with cold ethanol at a final concentration of 80 per cent. Low speed centrifugation was used to separate the ethanol-soluble from the insoluble components. The alcoholic extract was reduced in volume in a stream of filtered air and partitioned between water and chloroform to remove lipides and chlorophyll. The extracted amino acids and amides were separated by two-directional paper chromatography and quantitatively analyzed by procedures based on those outlined originally by Thompson and Steward (11), but as modified for later use in the Cornell laboratory. A solvent consisting of *n*-butyl alcohol—glacial acetic acid—water (9:1:2.9 v/v/v) replaced collidine and lutidine, and Whatman No. 3 paper replaced Whatman No. 1 in order to achieve both superior separations and to permit larger amounts to be applied to the paper. But, as necessary, use was also made of the solvent system which consisted of phenol-H<sub>2</sub>O-collidine-lutidine which gives superior results in part of the chromatogram. The factors and calibration curves which were used were those which applied to the method as standardized in the Cornell laboratories and which permit the various amino acids to be determined quantitatively. The accuracy of these procedures will not be discussed here since the relatively large differences to which attention will be directed are much greater than the agreement between replicated analyses.

## RESULTS

*The soluble amino acids and amides in the leaves of tomato plants which received complete nutrient solution with nitrogen supplied as nitrate or ammonium salts.* The leaves of tomato plants which had received nitrate as a component of a complete nutrient solution are distinguished by a high concentration of dicarboxylic amino acids, glutamic acid and aspartic acid often accounting for most of the amino nitrogen. Glutamine is the

dominant amide since asparagine was undetectable. The total soluble nitrogen is at a level far below that found in leaves supplied with ammonium nitrogen. Plants supplied with  $(\text{NH}_4)_2\text{SO}_4$  have an extremely high soluble nitrogen content with most of the nitrogen found in asparagine and glutamine. Table I depicts this situation with data selected from typical plants grown with nitrate or ammonium nitrogen.<sup>3</sup>

*Variations in the concentrations of the soluble nitrogen compounds.* A considerable range in the content of the soluble amino acids and amides in the leaves has been found. Data to illustrate this are compiled in Table II. For example, in the nitrate treatments, the total soluble nitrogen level ranged from 57 to 329  $\mu\text{g. N}$  per gram fresh weight. Among the individual constituents, glutamine varied from 0 to 40 per cent of the total nitrogen in ninhydrin reactive compounds, glutamic acid varied from 14 per cent to 64 per cent, aspartic acid from 8 per cent to 33 per cent, and  $\gamma$ -aminobutyric acid from 4 per cent to 13 per cent. The experiments are listed in Table II in the order of increasing concentration of total soluble nitrogen and the coefficients of correlation (correlation between the total N of soluble ninhydrin reactive compounds and the nitrogen of a particular compound) for each of the major amino compounds are also listed (7, p. 162). Glutamine, alanine, and serine have positive correlation coefficients that are significant at the 1 per cent level; glutamic acid and  $\gamma$ -aminobutyric acid are significant at the 5 per cent level, and no significant correlations were detected for aspartic acid and glycine. It is apparent, therefore, that the distribution of the amino compounds in the experiments is not random and that, in most instances, the level of the individual nitrogen compounds fluctuates with the level of the total soluble nitrogen.

The coefficients of correlation between the concentrations of individual amino acids in the leaves have been determined. The correlation between glutamine and alanine was +0.788 (significant at the 1 per cent level); between glutamic acid and alanine it was +0.731 (significant at the 5 per cent level). The coefficients of correlation between glutamine and glutamic acid, glutamic acid and  $\gamma$ -aminobutyric acid, glutamic acid and aspartic acid, and aspartic acid and alanine were not significant.

The variations in the concentrations of the components of the ammonium treatment (Table III) were of the same order with the major fluctuations residing in glutamine and the dicarboxylic amino acids. A relative decrease in glutamine content is accompanied by an increase in glutamic acid and, to a lesser degree, aspartic acid. Although the total soluble nitro-

<sup>3</sup> Only the major ninhydrin reacting compounds are reported in this table and in all those following; thus, at times, the per cent tabulation does not total 100. Threonine, valine, leucine(s), tryptophan, tyrosine, pipercolic acid, proline, and several unidentified compounds have been found to occur erratically and in very minor amounts. The total N in ninhydrin reactive compounds is the summation of the values obtained by the application of the ninhydrin reaction to those compounds which were present on the chromatograms.

TABLE I  
DISTRIBUTION OF NITROGEN IN LEAVES OF TOMATO PLANTS (PER CENT OF TOTAL NITROGEN IN NINHYDRIN REACTIVE COMPOUNDS)  
GROWN IN A COMPLETE NUTRIENT SOLUTION CONTAINING EITHER NITRATE OR AMMONIUM NITROGEN

| Form of<br>nitrogen supplied | Distribution of nitrogen expressed as per cent of the total nitrogen in<br>ninhydrin reactive compounds soluble in 80% ethanol |                  |        |            |         |           |                        | Total nitrogen in<br>ninhydrin reactive<br>compounds, $\mu\text{g./g.}$<br>fresh wt. |
|------------------------------|--------------------------------------------------------------------------------------------------------------------------------|------------------|--------|------------|---------|-----------|------------------------|--------------------------------------------------------------------------------------|
|                              | Aspartic<br>acid                                                                                                               | Glutamic<br>acid | Serine | Asparagine | Alanine | Glutamine | Arginine<br>and lysine |                                                                                      |
| Nitrate<br>Ammonium          | 14                                                                                                                             | 30               | 12     | 0          | 7       | 23        | 0                      | 11                                                                                   |
|                              | 6                                                                                                                              | 12               | 8      | 30         | 3       | 36        | 0                      | 5                                                                                    |



TABLE II

DISTRIBUTION OF NITROGEN IN THE LEAVES OF TOMATO PLANTS ( $\mu$ G. PER G. FRESH WEIGHT OF TOTAL NINHYDRIN REACTIVE COMPOUNDS) GROWN IN A COMPLETE NUTRIENT SOLUTION CONTAINING NITRATE NITROGEN

| Date of harvest                                       | Distribution of nitrogen expressed in $\mu$ g. nitrogen per g. fresh weight in ninhydrin reactive compounds soluble in 80% ethanol |               |          |         |          |           |                              | Total nitrogen in ninhydrin reactive compounds |
|-------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|---------------|----------|---------|----------|-----------|------------------------------|------------------------------------------------|
|                                                       | Aspartic acid                                                                                                                      | Glutamic acid | Serine   | Glycine | Alanine  | Glutamine | $\gamma$ -Amino-butyric acid |                                                |
| 5/27/54                                               | 14                                                                                                                                 | 26            | 2        | 0       | 2        | 6         | 7                            | 57                                             |
| 8/21/54                                               | 9                                                                                                                                  | 27            | 6        | 11      | 6        | 7         | 6                            | 70                                             |
| 8/6/53                                                | 15                                                                                                                                 | 50            | 0        | 0       | 8        | 0         | 6                            | 79                                             |
| 9/17/54                                               | 19                                                                                                                                 | 40            | 16       | 0       | 9        | 31        | 15                           | 133                                            |
| 8/20/53                                               | 22                                                                                                                                 | 68            | 0        | 0       | 12       | 34        | 10                           | 136                                            |
| 5/26/54                                               | 20                                                                                                                                 | 37            | 7        | 0       | 7        | 56        | 14                           | 141                                            |
| 9/18/54                                               | 25                                                                                                                                 | 48            | 10       | 0       | 10       | 32        | 12                           | 144                                            |
| 8/16/54                                               | 53                                                                                                                                 | 22            | 19       | 24      | 11       | 24        | 11                           | 163                                            |
| 8/26/54                                               | 13                                                                                                                                 | 54            | 20       | 11      | 15       | 54        | 17                           | 171                                            |
| 8/30/54                                               | 19                                                                                                                                 | 48            | 15       | 19      | 14       | 29        | 16                           | 171                                            |
| 8/20/54                                               | 36                                                                                                                                 | 72            | 72       | 0       | 30       | 82        | 16                           | 329                                            |
| r With total nitrogen in ninhydrin reactive compounds | +0.533                                                                                                                             | +0.606*       | +0.770** | +0.047  | +0.958** | +0.802**  | +0.652*                      |                                                |

\* Significant at 5% level; \*\* significant at 1% level.

r = The correlation coefficient between nitrogen in the compound in question and in the total nitrogen present in all the soluble compounds determined by ninhydrin.

gen content and the relative amounts of other constituents varied appreciably, the content of asparagine in the soluble nitrogen pool of ammonium treated plants was relatively stable (25 to 35 per cent).

Several experiments were conducted that involved the effects of trace elements upon plants recovering from nitrogen deficiency. It is, therefore, necessary to consider first the effects of nitrogen deficiency upon plants receiving a full complement of trace elements. The consequence of a period of nitrogen deficiency is to lower the level of soluble nitrogen in the leaves and, coincidentally, to produce a relatively higher concentration of asparagine. Recovery from a nitrogen deficient condition by supplying nitrate results in a decline in the level of asparagine, a temporary increase in the basic amino acids, a decline in glutamic acid, and an accumulation of glutamine and  $\gamma$ -aminobutyric acid (Table IV). It is clear that the decline in the level of asparagine and the temporary accumulation of the basic amino acids precedes the accumulation of glutamine. This sequence may be explained if asparagine accumulation is associated more with conditions of protein degradation (protein nitrogen acting as a reservoir of nitrogen for less complex nitrogenous constituents during a condition of low external nitrogen supply, the carbon moiety accumulating, in part, as asparagine) and if the glutamine level is more conducive to synthesis of protein and nucleic acid (5, 9). Upon the restoration of an external supply of nitrate nitrogen, the normal sequence of synthetic reactions that occur in leaves to produce protein and its associated nucleic acids is resumed, resulting in a decline of asparagine preceding the re-establishment of a high glutamine

TABLE III  
DISTRIBUTION OF NITROGEN IN THE LEAVES OF TOMATO PLANTS (PER CENT OF TOTAL NITROGEN IN NINHYDRIN REACTIVE COMPOUNDS)  
WHICH RECEIVED COMPLETE NUTRIENT SOLUTION CONTAINING AMMONIUM NITROGEN

| Date of harvest | Distribution of nitrogen expressed as per cent of the total nitrogen in ninhydrin reactive compounds soluble in 80% ethanol |               |        |         |            |         |           |        |          |                              | Total nitrogen in ninhydrin reactive compounds, $\mu\text{g./g.}$ fresh wt. |
|-----------------|-----------------------------------------------------------------------------------------------------------------------------|---------------|--------|---------|------------|---------|-----------|--------|----------|------------------------------|-----------------------------------------------------------------------------|
|                 | Aspartic acid                                                                                                               | Glutamic acid | Serine | Glycine | Asparagine | Alanine | Glutamine | Lysine | Arginine | $\gamma$ -Amino-butyric acid |                                                                             |
| 10/31/53        | 0                                                                                                                           | 1             | 5      | 0       | 34         | 0       | 50        | 3      | 6        | 1                            | 894                                                                         |
| 8/20/54         | 6                                                                                                                           | 12            | 8      | 0       | 30         | 3       | 36        | 0      | 0        | 5                            | 491                                                                         |
| 8/21/54         | 7                                                                                                                           | 13            | 4      | 2       | 25         | 5       | 37        | 0      | 0        | 7                            | 458                                                                         |

TABLE IV  
DISTRIBUTION OF NITROGEN IN TOMATO LEAVES ( $\mu\text{G.}$  OF NITROGEN PER G. FRESH WEIGHT IN NINHYDRIN REACTIVE COMPOUNDS) FROM PLANTS WHICH RECEIVED NUTRIENT SOLUTION CONTAINING NITRATE AFTER RECEIVING A NITROGEN DEFICIENT NUTRIENT SOLUTION FOR 6 DAYS

| Treatment                          | Distribution of nitrogen expressed in $\mu\text{g.}$ nitrogen per g. fresh weight in ninhydrin reactive compounds soluble in 80% ethanol |               |        |         |            |           |         |           |        |          | Total nitrogen in ninhydrin reactive compounds |
|------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|---------------|--------|---------|------------|-----------|---------|-----------|--------|----------|------------------------------------------------|
|                                    | Aspartic acid                                                                                                                            | Glutamic acid | Serine | Glycine | Asparagine | Threonine | Alanine | Glutamine | Lysine | Arginine | $\gamma$ -AB*                                  |
| 6 Days nitrogen deficient          | 2                                                                                                                                        | 5             | 5      | 2       | 8          | 2         | 6       | 16        | 0      | 0        | 16                                             |
| 2 Hours after addition of nitrate  | 5                                                                                                                                        | 0             | 7      | 5       | 11         | 3         | 13      | 18        | 9      | 11       | 28                                             |
| 6 Hours after addition of nitrate  | 6                                                                                                                                        | 0             | 6      | 4       | 16         | 1         | 14      | 27        | 12     | 17       | 28                                             |
| 24 Hours after addition of nitrate | 11                                                                                                                                       | 0             | 6      | 3       | 0          | 0         | 18      | 29        | 14     | 15       | 28                                             |
| 30 Hours after addition of nitrate | 16                                                                                                                                       | 0             | 16     | 13      | 11         | 3         | 29      | 113       | 0      | 0        | 52                                             |
|                                    |                                                                                                                                          |               |        |         |            |           |         |           |        |          | 70<br>130<br>143<br>140<br>262                 |

\*  $\gamma$ -Aminobutyric acid.

level. The temporary increase in the level of the basic amino acids may again precede their rapid use in the renewed synthesis of proteins.

The data presented in Table IV must be evaluated with consideration for the effects of the fluctuating environment and an accumulation of compounds in the plants from the nitrogen supplied in the nutrient media. The first harvest was performed at 9 A.M. with the harvests at 6 and 30 hours made at 3 P.M. The great increase in total nitrogen in ninhydrin reactive compounds between 24 and 30 hours would include effects of 6 hours of daylight and 30 hours of recovery from nitrogen deficiency. Plants are capable of accumulating large quantities of nitrate in their tissues and this reservoir of nitrogen would be readily assimilated, in the light, in particular. Table IV provides data typical of the four experiments of this type that were performed.

*The soluble nitrogen compounds in the roots of tomato plants which received complete nutrient solution with nitrogen supplied as nitrate or ammonium salts.* Tomato roots differ from the leaves in their lower total soluble nitrogen and in the presence of asparagine, lysine, and arginine as major constituents. As in leaves, the supply of  $\text{NH}_4^+$  produces an accumulation of soluble nitrogen, due almost entirely to the high amide level. The contrasting effects of nitrate and ammonium nutrition which were found in the leaves were also detected in the roots. The considerable range in the content of the nitrogenous compounds in the roots of tomato plants receiving nitrate nitrogen is depicted in Table V. Table V lists the coefficients of correlation ( $r$ ) of the nitrogen in individual amino compounds with total nitrogen of compounds detected by ninhydrin. All of the amino compounds (except glycine) have coefficients of correlation that are significant. It is probable that the variation in the levels of the individual amino compounds is a reflection of the levels of total soluble nitrogen. However, other factors are also operating because the coefficients of correlation of the nitrogen of pairs of individual amino compounds, viz., glutamic acid and glutamine (+0.749), glutamine and asparagine (+0.785), and glutamine and  $\gamma$ -aminobutyric acid (+0.894) are all significant at the 1 per cent level, whereas certain pairs such as aspartic acid and asparagine, aspartic acid and glutamic acid, and glutamic acid and  $\gamma$ -aminobutyric acid do not have significant coefficients of correlation. This suggests that these paired compounds were affected independently and in a different manner by the variables in question. A glance at the tables will show that factors also operated, other than those which determine total soluble nitrogen, to regulate the proportion of glutamic acid to glutamine, aspartic acid to asparagine, etc.

*Recovery of tomato plants from nitrogen deficiency as reflected in the amino acids in the roots.* Nitrogen deficient roots were conspicuous by their low nitrogen level, the absence of asparagine, and the accumulation of arginine.

TABLE V  
DISTRIBUTION OF NITROGEN IN ROOTS OF TOMATO PLANTS ( $\mu$ G. PER G. FRESH WEIGHT OF TOTAL NINHYDRIN REACTIVE COMPOUNDS)  
WHICH RECEIVED COMPLETE NUTRIENT SOLUTION CONTAINING NITRATE NITROGEN

| Date of harvest                                       | Distribution of nitrogen expressed as $\mu$ g. nitrogen per g. fresh weight in ninhydrin reactive compounds soluble in 80% ethanol |               |          |         |            |          |           |         |          |                              | Total nitrogen in ninhydrin reactive compounds |
|-------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|---------------|----------|---------|------------|----------|-----------|---------|----------|------------------------------|------------------------------------------------|
|                                                       | Aspartic acid                                                                                                                      | Glutamic acid | Serine   | Glycine | Asparagine | Alanine  | Glutamine | Lysine  | Arginine | $\gamma$ -Amino-butyric acid |                                                |
| 8/20/53                                               | 2                                                                                                                                  | 12            | 0        | 0       | 11         | 2        | 9         | 0       | 0        | 8                            | 45                                             |
| 8/16/54                                               | 3                                                                                                                                  | 13            | 2        | 1       | 0          | 3        | 6         | 3       | 11       | 3                            | 49                                             |
| 8/20/54                                               | 6                                                                                                                                  | 13            | 1        | 0       | 6          | 1        | 8         | 1       | 6        | 4                            | 49                                             |
| 6/2/54                                                | 6                                                                                                                                  | 15            | 3        | 0       | 8          | 5        | 5         | 2       | 3        | 6                            | 52                                             |
| 8/21/54                                               | 2                                                                                                                                  | 15            | 0        | 0       | 10         | 2        | 4         | 1       | 11       | 6                            | 55                                             |
| 8/14/53                                               | 4                                                                                                                                  | 9             | 0        | 10      | 8          | 5        | 15        | 0       | 0        | 9                            | 65                                             |
| 8/6/53                                                | 8                                                                                                                                  | 21            | 0        | 11      | 0          | 0        | 15        | 0       | 0        | 11                           | 67                                             |
| 8/9/54                                                | 8                                                                                                                                  | 14            | 3        | 7       | 0          | 7        | 5         | 4       | 7        | 3                            | 62                                             |
| 6/3/54                                                | 6                                                                                                                                  | 16            | 5        | 1       | 6          | 24       | 10        | 3       | 6        | 15                           | 97                                             |
| 8/24/54                                               | 4                                                                                                                                  | 16            | 4        | 0       | 20         | 20       | 19        | 4       | 20       | 19                           | 124                                            |
| 9/20/54                                               | 17                                                                                                                                 | 32            | 1        | 0       | 20         | 8        | 20        | 3       | 12       | 12                           | 129                                            |
| 9/21/54                                               | 11                                                                                                                                 | 45            | 3        | 2       | 15         | 11       | 21        | 2       | 15       | 17                           | 153                                            |
| 8/25/54                                               | 12                                                                                                                                 | 23            | 8        | 2       | 27         | 21       | 31        | 6       | 33       | 25                           | 192                                            |
| r With total nitrogen in ninhydrin reactive compounds | + .745**                                                                                                                           | + .690**      | + .739** | + .124  | + .824**   | + .853** | + .951**  | + .656* | + .842** | + .914**                     |                                                |

\* Significant at 5% level; \*\* significant at 1% level.

r = The correlation coefficient between nitrogen in the compound in question and in the total nitrogen present in all the soluble compounds determined by ninhydrin.



Feeding with nitrate produces a rapid synthesis of the soluble amino compounds and is reflected particularly in the early accumulation of glutamine and in the later accumulation of asparagine.

#### DISCUSSION

The features of the soluble nitrogenous compounds of the tomato plant, which are most intimately involved in protein metabolism and which may be detected by reaction with ninhydrin, are described in this paper. Particular attention is directed to the range of variation in concentration that may be encountered in plants exposed to full nutrient solution and to the contrasted effects of nitrate and ammonium as the sources of nitrogen.

The leaves of plants which received nitrate nitrogen were found to have less soluble amino nitrogen, less amide nitrogen, and more dicarboxylic amino acids than plants cultured with ammonium nitrogen. It has frequently been found that nitrate assimilation is related more directly to protein synthesis while ammonium is often associated more closely with an increase in soluble nitrogen compounds principally in the form of amides (9).

The roots of plants which received nitrate as a component of a complete nutrient solution differed from the leaves in less total soluble amino nitrogen and in the normal occurrence of asparagine, lysine, and arginine as important constituents of the pool of soluble compounds. Again, the effect of ammonium was to produce an accumulation of soluble nitrogen mainly in the form of amides.

A considerable range in the content of individual amino acids and amides in the soluble nitrogen fraction of tomato plants has been observed. Since these plants were grown under greenhouse conditions, i.e., in an essentially uncontrolled environment, the range of variation was to be expected and is to be attributed to intimate responses of the plants with features of the environment that were neither controlled nor identified at the time the experiments were carried out. The data reported, however, show the range of variation in composition of leaf and root that has to be appreciated, and they emphasize that the effects due to trace elements (Mn and Mo), which are still to be described in detail, need to be interpreted against this background. Since the alcohol soluble amino acid and amide composition of the tomato plant is so drastically affected by the form of nitrogen supplied ( $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) and since it may also be so much modified by the environmental conditions during growth, the effects which may be superimposed by the trace elements will need to be discerned. The experiments still to be described attempted to do this.

The variation in the levels of the amino compounds in the leaves and roots is not of a random nature since the correlation between the amino compounds and total soluble amino acid and amide nitrogen is positive and significant for all the compounds in the roots and all in the leaves with

the exception of aspartic acid and glycine. Thus the cause of the fluctuations which occurred in the various experiments may be attributed first to factors that affect the size of the pool of soluble nitrogen compounds as a whole as this is influenced by differences in the age of the plants, in light intensity, night temperature, and daily duration of light. Since work with the necessary degree of environmental control to delineate the effects of these variables individually was not done, reference may be made to experiments which do show the influence of environment upon the metabolism of amino acids in plants. Work with the mint plant (2, 6, 8) has shown the magnitude and rapidity of the response of this plant to changes in the environment (day length, night temperature, and nutrition). The main site where these variables operate is now believed to be the keto acid fraction which links carbohydrate and nitrogen metabolism, and the overriding variable seems to be the effect of night temperature (8).

In the work described here and to be described, the emphasis is being placed upon certain nutritional factors ( $\text{NO}_3^-$  vs.  $\text{NH}_4^+$ , and effects due to Mn and Mo) but it is recognized that, had the experimental design of conditions allowed, many of the recorded differences could, no doubt, have been correlated with environmental factors that were uncontrolled. These environmental factors had effects which were apparently of two types. The major effect was upon the total soluble amino acid and amide nitrogen. Almost all the amino compounds were found to fluctuate with the level of total soluble nitrogen as revealed by the ninhydrin reaction. However, another secondary effect is upon the interrelationships between the individual compounds.

The immediate consequences of nitrogen deficiency in the tomato plant are manifest in the marked decrease in soluble nitrogen and, in particular, in glutamine in the leaves and asparagine in the roots. The recovery of plants, which had been grown in nutrient solutions deficient in nitrogen and after they have been supplied with nitrate nitrogen, results in an increase in soluble nitrogen with emphasis upon glutamine, glutamic and aspartic acids; recovery in the presence of ammonium nitrogen produces its effect primarily upon the increased level of glutamine.

This first paper, therefore, presents the background of knowledge of the nitrogen compounds of tomato plants showing how they respond to experimental conditions of the kind adopted for the later study of manganese and molybdenum effects. The experimental results relate to tomato plants receiving a complete complement of trace elements and either nitrate or ammonium as the nitrogen sources. The range of effects due to environmental fluctuations, age of plants, and manipulation of the nutrient solution, has been discussed and the effects noted. It is now possible to present the superimposed effects of manganese and molybdenum with the emphasis placed upon the effects which are attributable to the concomitant nitrogen supply and to the way this interacts with each of the trace elements in question.

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# ANATOMICAL AND MORPHOLOGICAL COMPARISONS OF NORMAL AND PHYSIOLOGICALLY DWARFED SEEDLINGS OF RHODOTYPOS TETRAPETALA AND PRUNUS PERSICA<sup>1</sup>

MYRON C. LEDBETTER<sup>2</sup>

## SUMMARY

An analysis was made of the cell development in seedlings of *Rhodotypos tetrapetala* exhibiting physiological dwarfing, a condition associated with failure of the internodes to elongate normally. The number of cells and their size and shape was determined in six-week-old seedlings grown alongside each other in the greenhouse from unchilled seed, chilled seed, and chilled seed treated with maleic hydrazide at 10 p.p.m. for 70 hours before planting to inhibit growth.

The first internodes of dwarf seedlings from unchilled seed had fewer cells than normal seedlings from chilled seed, but the volume of individual cells in the epidermis and cortex was very similar. In two series of experiments there were approximately 6 times as many epidermal cells, 4 times as many outer cortical cells, 4.8 times as many inner cortical cells, and 4.2 times as many pith cells in the first internode of the normal plants as in the dwarf seedlings. The difference in size of individual cells was much less conspicuous although there were some significant differences in shape of the cells. The volume per cell in the epidermis, outer cortex and inner cortex was essentially the same. However, pith cells of normal plants were 3.6 times larger than in dwarfed plants.

The internode length showed significant partial correlation coefficients with the number of cells in the pith of 0.604, and with the size of the pith cells of 0.616. This suggests that the failure of pith cells to divide may be the predominant influence in instigating the dwarf habit of growth. Internode shape had significant partial correlation coefficients with shape of the cells of the inner cortex of 0.809, and with the shape of the cells of the pith of 0.673.

Comparable studies on cell composition of the second, third, and fourth internodes above the cotyledons revealed a similar state in which cell division appeared to have been subnormal in the physiologically dwarfed seedlings.

The inhibition of internode elongation by maleic hydrazide resulted in several changes analogous to physiological dwarfing. The number of cells in the pith and their mean volume were reduced slightly more than in the physiologically dwarfed seedlings. The number of epidermal cells was reduced somewhat more than in the dwarfs but the differences were not significant.

The principal anatomical change during the chilling process to break dormancy of *Prunus persica* was the development of leaf primordia. These primordia probably are directly concerned with regulation of the dormancy responsible for physiological dwarfing. When leaf primordia were removed from *Rhodotypos* seedlings which were developing normally from chilled seed the internodes failed to continue to elongate. This suggests that there resides in the leaf primordia some principle which either promotes cell multiplication or else neutralizes an inhibitor of cell development.

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## INTRODUCTION

The dormancy of certain rosaceous seeds such as those of *Rhodotypos tetrapetala* (Sieb.) Makino (*R. kerrioides* Sieb. & Zucc.) (jethead) and *Prunus persica* (L.) Batsch (peach) can be broken by chilling while in a moist medium at approximately 5° C. for 8 to 10 weeks. If the embryos of dormant seed are excised and placed under normal conditions for growth they will germinate but the seedlings fail to grow normally and produce plants commonly referred to as physiological dwarfs (13, pp. 135-136). The internodes of these seedlings fail to elongate and many of the leaves are deformed (14, 20, 21, 50, 51). This incomplete dormancy usually persists for years unless the seedlings are chilled.

The factors responsible for physiological dwarfing are not well understood. The character of subsequent growth is determined in the scion as shown by the reciprocal grafts between normal and dwarf parts of the peach by Flemion and Waterbury (26). The process is analogous to the failure of buds to develop normally on trees that have not been exposed to low temperature during the winter as pointed out by Crocker (12, pp. 94-96).

The question arises as to whether there has been a failure of the embryonic cells to divide freely or merely a suppression of cell enlargement in the stem tissues of the physiological dwarfs. There is substantial evidence that slow growing or small varieties of plants have fewer cells in the leaves and stems than large varieties of the same species as reported by Ashby and Wangermann (4, 5), Arney (2), Ullrich (52), Njoku (42), and Sinnott (45). A similar condition was found in fruits of several varieties of tomatoes and their hybrids by Houghtaling (35) and of cucurbits by Sinnott (46), although the larger varieties frequently had larger cells as well. Leaf size was not necessarily correlated with size of cells in a series of leaves from the same branch examined by Ullrich (52).

On the other hand, cell size seems to determine maximum growth potential in some organs affected by environmental conditions. The larger leaves produced by topped tobacco plants are largely associated with an increase in size of cells according to Avery (6). Variations in the balance of minerals apparently affect the form of leaves of *Potamogeton* by changing the cell shape (43).

Since the altered growth habits in dwarfed seedlings might be due to changes in cell division, cell enlargement or cell shape, comparative studies were made of the stem tissues of normal and dwarf plants and normal plants inhibited by maleic hydrazide. The anatomical changes in tissues of peach during the breaking of dormancy by chilling were examined to see which organs began growth and whether the regulatory factor for elongation resided in these tissues. The results of these observations are summarized below.

## MATERIALS AND METHODS

Most of the studies were made on seedlings of jetbead in preference to peach since the plant material was much more uniform in growth habit. The seed of jetbead was obtained from ripe fruits harvested at Yonkers, N. Y. and stored at room temperature. These produced very uniform seedlings when chilled to break dormancy. Peach pits of the cultivar Lovell were obtained from a commercial source in California and stored under dry conditions at  $1^{\circ}\text{C}$ . to maintain vitality. Preparatory to setting up the various experiments the hard endocarps were removed with the aid of a machine devised for this purpose (22) before beginning the chilling process to break dormancy.

The recommended procedure (19) of treating seed to break dormancy of jetbead was followed except that pretreatment in moist granulated peat moss at  $30^{\circ}\text{C}$ . to soften the endocarp before chilling was omitted. The seed were placed in moist Vermiculite at weekly alternating temperatures of  $5^{\circ}$  and  $10^{\circ}\text{C}$ . for 6 weeks or longer. The chilled seed were then planted in composted soil under greenhouse conditions. Embryos were excised from untreated seed and germinated on moist filter paper at room temperature for about one week and the seedlings were then transplanted to the greenhouse alongside the treated lot.

Three experiments were undertaken with jetbead to determine the cell composition of stem tissues. In the first experiment, made during January, seedlings from excised embryos and chilled seed were held for six weeks. Five dwarf and ten normal seedlings were measured and sections were taken from the first internode of each for histological examination. The second experiment was similar to the preceding except the plants were grown during August. Sections were taken when they were four weeks old and examinations were made of only the pith and epidermis from the first, second, third, and fourth internodes above the cotyledons. The third experiment, conducted during June, was similar to the second one except that the plants were grown for 5 weeks and included seedlings grown from chilled seed, chilled seed soaked in 10 p.p.m. of maleic hydrazide for 70 hours to inhibit growth, and excised embryos of dormant seed.

In addition to these three experiments auxiliary observations were made on the extraxylary fibers and vessel elements of normal and dwarf seedlings grown for 7 and 13 weeks in the greenhouse, on the length of leaves in relation to length of the internode below them, on the size and number of epidermal cells of the leaf in relation to size of the leaf, and on the development of normal seedlings after removal of the leaf primordia. In the latter experiments dormancy was broken by chilling 150 seed for 13 weeks at alternating temperatures of  $5^{\circ}$  and  $10^{\circ}\text{C}$ . They were divided into two uniform lots. In one lot the first pair of leaf primordia was broken off by a needle while the plants were held under a stereoscopic microscope;

seedlings of the second lot were left intact. The seedlings were transplanted to soil and held in the greenhouse for 5 weeks. The length of the first four internodes was measured to the nearest millimeter in 26 control plants and 13 from which the first pair of leaf primordia had been removed.

One experiment was made with peaches to determine the anatomical changes during the breaking of dormancy by chilling. Seed were placed in moist granulated peat moss and held at 5° C. for 98 days. Samples of three or four seed each were removed at 10, 40, 68, and 98 days. The epicotyl was placed in fixative and sectioned.

#### MICROTECHNIQUE

All material to be sectioned was killed and fixed in Craff II or V (44, p. 18), and dehydrated in dioxane (44, p. 28). Craff II was used for the immature parts, and Craff V for the mature stem material. Much of the stem material was too brittle to yield unbroken sections when embedded in rubberized paraffin. It was found that a modification of the *n*-butyl methacrylate method De Giusti and Ezman recommend for arthropods (15) facilitated sectioning of the refractory materials. The following deviations from their method were made. The material to be sectioned was fixed in one of the Craff solutions, dehydrated in dioxane, placed in a 1:1 (v/v) mixture of dioxane—methacrylate monomer for a minimum of 4 hours, and left in pure monomer overnight or longer at room temperature. A 1:2 (v/v) mixture of Tissuemat—monomer was the most satisfactory embedding material from which sections of these internodes could be cut. This method was also found useful in making thin (5  $\mu$ ) sections of jetbead shoot apices. Serial sections of the peach epicotyls at 10  $\mu$  and stems of jetbead at 15 to 20  $\mu$  were cut on a rotary microtome.

Several combinations of stains were tested and used. For the peach epicotyls Harris' hematoxylin and Safranin O were used. Internode sections cut from rubberized paraffin were stained with Feulgen's stain and Fast Green FCF. Besides the nuclei the Feulgen reaction also stained lignified cell walls and the cuticle (40). Sections cut from the *n*-butyl methacrylate preparations were more difficult to stain. For these sections Harris' hematoxylin and Acid Fuchsin were satisfactory as were Safranin O and Crystal Violet, the Crystal Violet being made up in xylene as described by Johansen (37, p. 57).

Portions of the wood or bark to be macerated were placed in Jeffrey's solution (37, p. 104) overnight and washed thoroughly. Best results were obtained by staining the treated and washed pieces with Basic Fuchsin and dehydrating them before maceration. Maceration was then accomplished by teasing the cells apart with needles while the fragments were mounted in balsam damar.

Leaf cells were examined in cleared material. The leaves were dipped

briefly into boiling water and then treated with 85 per cent lactic acid at  $100^{\circ}$  C. until cleared. Projection drawings of the upper epidermis were made from portions of the leaves mounted on microscope slides in 85 per cent lactic acid.

#### CELL MEASUREMENTS

*Drawings.* Cell dimensions of the epidermis, two cortical layers and pith were measured from drawings, which were made as nearly as possible from the central portion along the length of the internode. Drawings of cell outlines were made by projecting the image of the prepared slide through the microscope to the paper using a carbon arc as a light source. A prism and large first-surface mirror were used with the microscope for convenient placement of the image on the drawing paper. Areas were measured with a planimeter which was standardized for each magnification.

The areas of the internode, epidermis, and cortical layers were calculated from the product of their circumferences and internode length. The volumes of the internode and pith were calculated from the product of their cross sectional areas and the internode length.

*Epidermal and cortical layers.* The size and number of epidermal cells were determined by two methods. The first method was used for the cortical cells as well. The size of the cells was calculated from the product of cell length and width. The number of cells was found from the quotient of the area of the entire cell layer and the average size of cells. The average cell length was determined from median longitudinal stem sections, noting the number of cells found in a measured file of cells. For any one internode these measurements were combined and the average cell length obtained. The tangential width of the cells was found from cross sections by noting the number of cells in a measured quadrant.

The epidermal cell size was measured by a second method. Drawings were made from longitudinal tangential sections of the epidermis. It was possible to match the projection of successive sections over drawings of previous sections and to build up an area representing a large layer of epidermis. From the area of the cell group drawn and the number of cells, the average size of the epidermal cells was determined. The number of cells was then the quotient of the area of the cell layer and the average epidermal cell size in tangential projection. The stomata and their surrounding cells if distorted were excluded from these measurements in both methods.

*Pith.* The pith cell volume was determined from the product of the average cell length and its cross sectional area. The cell number was found from the quotient of the pith volume and the average cell volume. The pith cells form well-defined files in jetbead. The length of the cells was found from these cell files by the same method used for the epidermal and cortical cells. The area of the pith included in the measurement was



arbitrarily limited because there is no sharp demarcation between the pith and the xylem. This area was a quadrangle, two vertices of which were at the patches of small thin-walled parenchyma cells found at the edge of the pith at the inner portion of the vascular traces which connect to the two leaves at the node above. A line was drawn between these two points. The two other vertices were at the intersection with the xylem of a second line which was perpendicular to the first at its midpoint. The cells with their very small intercellular spaces which were completely within the quadrangle were included in the measurements. The intercellular spaces accounted for a small portion of the total area measured.

*Vessel elements and extraxylary fibers.* Measurements of the length of the vessel elements and fibers were made with an ocular micrometer. Extraxylary fibers were measured from tip to tip. Only those fibers which were unbroken and completely separated from each other were used. The length of vessel elements was taken as the distance between the centers of the simple perforations of their end walls. This represents the length of the cambial initials from which they arise since the vessels are formed from longitudinal series of cells. Tip growth is thus excluded.

*Statistical methods.* The significance of differences between samples was tested by the *t*-test and the tables of Snedecor (47). Small samples were checked by Wilcoxon's rank method (47, p. 116) for values about which there might be some doubt. Partial correlation coefficients and their significance were found by the usual methods (18; 27, pp. 144-149).

## RESULTS AND OBSERVATIONS

*Comparisons of first internodes.* In the first experiment with jetbead the seedlings were grown in the short day length of winter until their fourth pair of true leaves unfolded. The 10 normal and 5 dwarf plants were found to be in the 7th or 8th or more rarely the 6th or 9th plastochron as determined by counting the total number of leaves and leaf primordia above the cotyledons. There was no significant difference between the plastochron age of the normal and dwarf plants at the end of the experiment (6 weeks). However, the internodes of the normal plants were about 4 times as long, 1.75 as large in cross section, and had 8 times as much volume (Table I). The epidermal cell development was determined by examination of cells in tangential sections of the first internode (Method 1) or in cross section (Method 2). The results of the two methods were in close agreement. The epidermis of the normal plants had about 5.8 times the area with cells of about the same area but 6.3 times as numerous as that of the dwarf plants. These cells of the normal plants were 0.8 times as wide as those of the dwarf plants. The first two layers of cortical cells immediately below the epidermis were designated cortex I and cortex II respectively. These two layers were very similar, and those of the normal had about

5.3 times the area made up of cells which were approximately the same area with 4.4 times as many cells. The cortical cells of the normal plants were 1.5 times as long and 0.8 times as wide as those of the dwarf. The volume of the pith of the normal plants was about 11.5 times that of the dwarf plants with cells 3.6 times as large and 4.2 times as numerous. The pith cells of the normal plants were both longer (1.5 times the length) and wider (2.5 times the cross sectional area) than those of the dwarf.

In two auxiliary experiments to the above, the bark and wood of the first internode of jetbead seedlings were macerated in order to make measurements on the vessel elements and extraxylary fibers. The vessel elements that were used in these measurements had pitted walls and were presumably from secondary xylem. Spiral secondary thickenings were found in the early primary xylem vessels of the normal and dwarf plants. The extraxylary fibers are grouped exterior to the phloem and are probably primary phloem fibers as described by Esau (17, pp. 199, 201). Their tips were often branched and forked, showing that growth between and around other cells had taken place; thus their length includes the intrusive growth at the tips in addition to the length of the cell initial, and the symplastic growth with the expansion of the whole internode. In one of these experiments 9 normal and 10 dwarf plants were grown in December and January for 7 weeks. The first internodes of these normal plants were 4.5 times as long as those of the dwarfs, and had vessel elements which were 1.7 times as long with 2.75 times as many elements per internode length (Table I). The bark of these plants was not successfully separated from the wood. In another experiment 9 normal and 6 dwarf plants were grown for 13 weeks ending in April in order to allow time for the bark to develop. The first internodes of these normal plants were 2.3 times as long as those of the dwarf with vessel elements 1.2 times as long and 1.9 times as many elements per vessel. The extraxylary fibers of these normal plants were 1.5 times as long as those of the dwarfs.

*Partial correlations between internode characters and cell characters.* Partial correlation coefficients for a number of characters of the first internode of jetbead were computed from the measurements made in the first experiment. In computing these correlations the normal and dwarf plants were treated as one continuous series. This is a valid procedure since scatter diagrams constructed for several of the correlated factors showed a continuous pattern for the group as a whole. The partial correlation coefficients are presented in Table II. In the four sections of this table the partial correlations were computed between an internode character and a cellular character for the epidermis, two cortical layers and the pith, in each case holding the particular cell character constant for the other three tissues. Significant partial correlations were found between the following: internode length and the number of pith cells (0.604), internode length and

TABLE I  
MEAN VALUES FOR FIRST INTERNODES AND THEIR CELLS FROM NORMAL AND  
PHYSIOLOGICALLY DWARFED SEEDLINGS OF JETBEAD AND THE RATIO OF  
MEASUREMENTS FOR NORMAL/PHYSIOLOGICAL DWARF

| Method I                                                                                                                                            |        |       |         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|--------|-------|---------|
| Normal plants from seed chilled 16 wks., normal and physiologically dwarfed plants grown in greenhouse 6 wks. Cell area from tangential projection. |        |       |         |
|                                                                                                                                                     | Normal | Dwarf | N/D     |
| Internode                                                                                                                                           |        |       |         |
| Length (mm.)                                                                                                                                        | 14.25  | 3.09  | 4.61**  |
| Area cross section ( $\mu^2 \times 10^{-5}$ )                                                                                                       | 7.57   | 4.62  | 1.63**  |
| Volume ( $\mu^3 \times 10^{-9}$ )                                                                                                                   | 10.64  | 1.25  | 8.51**  |
| Epidermis                                                                                                                                           |        |       |         |
| Area cell layer ( $\mu^2 \times 10^{-6}$ )                                                                                                          | 43.43  | 6.91  | 6.29**  |
| Area per cell ( $\mu^2$ )                                                                                                                           | 1675   | 1426  | 1.18    |
| Cell number ( $\times 10^{-3}$ )                                                                                                                    | 30.82  | 4.59  | 6.71**  |
| Method II                                                                                                                                           |        |       |         |
| Same as Method I except all cell measurements from median longitudinal and cross sections.                                                          |        |       |         |
| Internode                                                                                                                                           |        |       |         |
| Length (mm.)                                                                                                                                        | 14.25  | 3.89  | 3.66**  |
| Area cross section ( $\mu^2 \times 10^{-5}$ )                                                                                                       | 7.97   | 4.33  | 1.84**  |
| Volume ( $\mu^3 \times 10^{-9}$ )                                                                                                                   | 11.32  | 1.51  | 7.49**  |
| Epidermis                                                                                                                                           |        |       |         |
| Area cell layer ( $\mu^2 \times 10^{-6}$ )                                                                                                          | 44.7   | 8.50  | 5.26**  |
| Cell length ( $\mu$ )                                                                                                                               | 56.6   | 45.2  | 1.25    |
| Cell width tangential ( $\mu$ )                                                                                                                     | 22.6   | 28.3  | 0.798** |
| Cell length $\times$ width ( $\mu^2$ )                                                                                                              | 1253   | 1162  | 1.08    |
| Cell number ( $\times 10^{-3}$ )                                                                                                                    | 41.10  | 7.05  | 5.83**  |
| Cortex I, First layer of cortical cells below epidermis                                                                                             |        |       |         |
| Area cell layer ( $\mu^2 \times 10^{-6}$ )                                                                                                          | 41.97  | 8.10  | 5.18**  |
| Cell length ( $\mu$ )                                                                                                                               | 41.29  | 26.98 | 1.53*   |
| Cell width tangential ( $\mu$ )                                                                                                                     | 20.21  | 24.56 | 0.822*  |
| Cell length $\times$ width ( $\mu^2$ )                                                                                                              | 839.7  | 640.4 | 1.31    |
| Cell number ( $\times 10^{-3}$ )                                                                                                                    | 54.56  | 13.47 | 4.05**  |
| Cortex II, Second layer of cortical cells below epidermis                                                                                           |        |       |         |
| Area cell layer ( $\mu^2 \times 10^{-6}$ )                                                                                                          | 41.16  | 7.71  | 5.34**  |
| Cell length ( $\mu$ )                                                                                                                               | 44.88  | 29.06 | 1.54**  |
| Cell width tangential ( $\mu$ )                                                                                                                     | 21.28  | 29.50 | 0.721** |
| Cell length $\times$ width ( $\mu^2$ )                                                                                                              | 961.2  | 806.0 | 1.19    |
| Cell number ( $\times 10^{-3}$ )                                                                                                                    | 45.64  | 9.56  | 4.77**  |

\* Significant difference between values normal and dwarf plants at 5 per cent level based on *t*-test.

\*\* Significant difference between values normal and dwarf plants at 1 per cent level based on *t*-test.

TABLE I—(Continued)

|                                                                                                                                                    | Normal | Dwarf | N/D    |
|----------------------------------------------------------------------------------------------------------------------------------------------------|--------|-------|--------|
| <b>Pith</b>                                                                                                                                        |        |       |        |
| Volume ( $\mu^3 \times 10^{-9}$ )                                                                                                                  | 1.946  | 0.169 | 11.5** |
| Cell length ( $\mu$ )                                                                                                                              | 56.1   | 38.0  | 1.48** |
| Cell area cross section ( $\mu^2$ )                                                                                                                | 969    | 392   | 2.47** |
| Cell volume ( $\mu^3 \times 10^{-3}$ )                                                                                                             | 53.7   | 14.9  | 3.60** |
| Cell number ( $\times 10^{-3}$ )                                                                                                                   | 36.9   | 8.85  | 4.17** |
| Normal plants from seed chilled 18 wks., normal and physiologically dwarfed seedlings grown in greenhouse 7 wks. Cell measurements from macerates. |        |       |        |
| <b>Internode</b>                                                                                                                                   |        |       |        |
| Length (mm.)                                                                                                                                       | 14.44  | 3.19  | 4.53** |
| <b>Vessel elements</b>                                                                                                                             |        |       |        |
| Cell length† ( $\mu$ )                                                                                                                             | 278.6  | 166.7 | 1.67** |
| Cells/internode length                                                                                                                             | 53.38  | 19.43 | 2.75** |
| Normal plants from seed chilled 6 wks., normal and physiologically dwarfed seedlings grown in greenhouse 13 wks. Cell measurements from macerates. |        |       |        |
| <b>Internode</b>                                                                                                                                   |        |       |        |
| Length (mm.)                                                                                                                                       | 7.50   | 3.21  | 2.34** |
| <b>Vessel elements</b>                                                                                                                             |        |       |        |
| Cell length† ( $\mu$ )                                                                                                                             | 220.6  | 182.4 | 1.21** |
| Cells/internode length                                                                                                                             | 33.72  | 17.42 | 1.94** |
| <b>Extraxylary fibers</b>                                                                                                                          |        |       |        |
| Fiber length ( $\mu$ )                                                                                                                             | 532.0  | 347.8 | 1.53** |

† Measured between centers of perforations.

the size of the pith cells (0.616), internode shape and the shape of the inner cortical cells (0.809), and internode shape and the shape of the pith cells (0.673). There was no significant partial correlation between internode length and the length of the cells.

In another experiment five normal and eight dwarf seedlings were used to find the simple correlation of leaf area to the size and number of cells of the first pair of leaves. The area of the leaves used ranged from 9 to 435 mm.<sup>2</sup>. The correlation between leaf area and epidermal cell number was highly significant with a coefficient of 0.981. There was no correlation between leaf size and epidermal cell size. There appeared to be little difference in cell size regardless of the size of the leaf in the palisade, spongy parenchyma or lower epidermis as seen by microscopic examination of these cleared leaves.

*First four internodes.* In the second experiment the first four internodes above the cotyledons of normal and dwarfed jethead seedlings were examined to see if the anatomical differences between normal and dwarf



TABLE II

PARTIAL CORRELATION COEFFICIENTS OF INTERNODE AND CELL CHARACTERS IN JETBEAD.  
NORMAL AND DWARF PLANTS TREATED AS ONE GROUP

| Characters correlated                   | Partial coefficients |
|-----------------------------------------|----------------------|
| Internode length:epidermal cell number  | -0.539               |
| Internode length:cortex I cell number   | 0.153                |
| Internode length:cortex II cell number  | 0.337                |
| Internode length:pith cell number       | 0.604*               |
| Internode length:epidermal cell size†   | 0.002                |
| Internode length: cortex I cell size†   | 0.010                |
| Internode length:cortex II cell size†   | 0.003                |
| Internode length:pith cell size††       | 0.616*               |
| Internode length:epidermal cell length  | 0.134                |
| Internode length:cortex I cell length   | 0.018                |
| Internode length:cortex II cell length  | 0.061                |
| Internode length: pith cell length      | 0.228                |
| Internode shape‡:epidermal cell shape‡‡ | -0.381               |
| Internode shape‡:cortex I cell shape‡‡  | -0.441               |
| Internode shape‡:cortex II cell shape‡‡ | 0.809**              |
| Internode shape‡:pith cell shape‡       | 0.673*               |

\* Significant correlation between characters at 5 per cent level.

\*\* Significant correlation between characters at 1 per cent level.

† Size = length  $\times$  tangential width.†† Size = length  $\times$  mean width.‡ Shape = length  $\div$  mean diameter.‡‡ Shape = length  $\div$  tangential width.

seedlings found for the first internode (Table I) apply to the stem as a whole. Two normal seedlings were used from seed which had been chilled 14 weeks and two dwarfed seedlings from unchilled seed. The internodes were measured and fixed when the fourth pair of leaves was unfolded after four weeks' growth in the greenhouse during August. At this time the first three internodes had completed their elongation but the fourth had not. The epidermis and pith showed the extremes in most cell characters measured for the first internode, and were used to represent the cellular changes in the internode as a whole. Measurements from projection drawings of epidermal cells were made from tangential sections and those of the pith from cross and median longitudinal sections. The results are presented in Figure 1. The sections from only one of the second internodes of the dwarf seedlings were usable, which accounts for the inconsistent results in cell number and size at internode number two in this graph. The internodes of the normal seedlings were 3.5 times as long as those of the dwarf for the first internode and about 1.8 times as long in the third and fourth internodes. The normal seedlings contained 4.1 times as many pith cells as the dwarf in the first internode and about 2.1 times as many in the third and fourth internodes. There were 3.3 times as many epidermal cells in the first internode of the normal as in the dwarf and about 2.5 times as

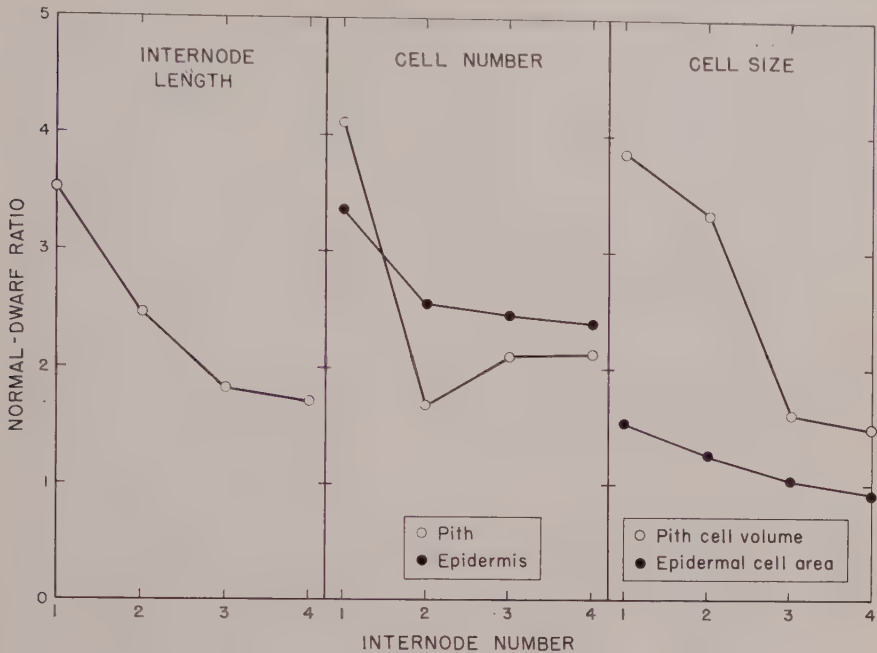


FIGURE 1. Internode length, cell number and cell size in successive internodes of normal and physiological dwarfed jetbead.

many in the third and fourth internodes. The pith cells of the normal seedlings were 3.8 times as large as those of the dwarf in the first internode and about 2.5 times as large in the third and fourth internodes. The epidermal cells of the normal seedlings were only 1.5 times as large as those of the dwarf in the first internode and were approximately the same size in the third and fourth internodes.

*Maleic hydrazide stunting.* In the third experiment with jetbead the seedlings were grown in June under light-tight covers to restrict light to about eight hours per day. The imposed short day was used in this experiment to assure that the plants from the unchilled seed would be dwarfed (38). The treatment with maleic hydrazide was at 10 p.p.m. for 70 hours. Shorter treatment or lower concentrations failed to affect internode length, while higher concentrations (30 p.p.m.) killed the embryos. The high sensitivity of these embryos probably is due to their young age (28). Four plants each were used from normal seedlings, those stunted with maleic hydrazide, and the physiological dwarfs. The epidermis and pith of the first internodes were examined and the results are shown in Table III. The values found for the first internodes of normal and dwarfed seedlings were similar to those in the first experiment. In this case the internodes of the normal plants were 2.6 times as long, 1.9 times as large in cross sectional

TABLE III  
MEAN VALUES FOR FIRST INTERNODES AND THEIR EPIDERMAL AND PITH CELLS FROM NORMAL, PHYSIOLOGICAL DWARFS AND MALEIC HYDRAZIDE-TREATED CHILLED EMBRYOS OF JETBEAD, THEIR RATIOS TAKEN IN PAIRS

|                                               | Normal† | Dwarf†† | MH‡    | N/D    | N/MH   | D/MH   |
|-----------------------------------------------|---------|---------|--------|--------|--------|--------|
| Internode                                     |         |         |        |        |        |        |
| Length (mm.)                                  | 10.18   | 3.90    | 1.15   | 2.61** | 8.85** | 3.39** |
| Area cross section ( $\mu^2 \times 10^{-3}$ ) | 736.0   | 381.2   | 360.5  | 1.93** | 2.04** | 1.06   |
| Volume ( $\mu^3 \times 10^{-9}$ )             | 7.473   | 1.501   | 0.4133 | 4.98** | 18.1** | 3.03*  |
| Epidermis                                     |         |         |        |        |        |        |
| Area cell layer ( $\mu^2 \times 10^{-6}$ )    | 30.80   | 8.53    | 2.44   | 3.61** | 12.6** | 3.40** |
| Area per cell ( $\mu^2$ )                     | 981.0   | 1580    | 533.3  | 0.617  | 1.84** | 2.08*  |
| Cell number ( $\times 10^{-3}$ )              | 31.83   | 6.15    | 4.63   | 5.18** | 6.88** | 1.33   |
| Pith                                          |         |         |        |        |        |        |
| Volume ( $\mu^3 \times 10^{-6}$ )             | 1271    | 84.0    | 48.6   | 15.1** | 26.1** | 1.73   |
| Cell length ( $\mu$ )                         | 47.70   | 32.63   | 28.48  | 1.46*  | 1.68** | 1.15   |
| Cell area cross section ( $\mu^2$ )           | 1053    | 302.5   | 334.5  | 3.48** | 3.15** | 0.95   |
| Cell volume ( $\mu^3 \times 10^{-3}$ )        | 50.40   | 10.22   | 9.92   | 4.93** | 5.08** | 1.03   |
| Cell number ( $\times 10^{-3}$ )              | 27.65   | 8.69    | 5.01   | 3.18** | 5.52** | 1.73   |

\* Significant difference between pairs at 5 per cent level, based on *t*-test.

\*\* Significant difference between pairs at 1 per cent level, based on *t*-test.

† After-ripened 8 wks. at low temperature.

†† Non-after-ripened.

‡ MH = Maleic hydrazide-treated for 70 hrs. in 10 p.p.m. as diethanolamine salt by weight.

area, and had 5 times the volume of those of the dwarf. The epidermis covered 3.6 times the area and had cells 5.2 times as numerous and somewhat smaller, but not significantly so. The pith had 15.1 times the volume of the internodes of the dwarf seedlings, with cells 1.5 times as long, 3.5 times as large in cross section, 4.9 times the volume, and 3.2 times as numerous.

The maleic hydrazide-stunted seedlings had internodes which were even smaller than the physiological dwarfs. The normal internodes were 8.9 times as long as those of the maleic hydrazide-stunted plants, with 2.0 times the cross sectional area and 18.1 times the volume. The epidermis of the normal seedlings had 12.6 times the area with cells 1.8 times as large in area, and 6.9 times as numerous. The pith of the normal seedlings was 26.1 times as large in volume with cells 1.7 times as long, 3.2 times as large in cross section, 5.1 times the volume, and 5.5 times as numerous.

The internodes of the maleic hydrazide-stunted seedlings were more like the physiologically dwarfed internodes than those of the normal plants. The internodes of physiologically dwarfed seedlings were 3.4 times as long as those stunted by maleic hydrazide with about the same cross sectional area, and 3.6 times the volume. The epidermis of the internodes of physiologically dwarfed seedlings was 3.5 times as large in area, with cells 3.0 times as large in area, and of about the same number. There were no significant differences in pith characters between internodes of physiologically dwarfed seedlings and those of maleic hydrazide-stunted plants.

Using Wilcoxon's rank method the differences between the physiological dwarf and the maleic hydrazide-stunted seedlings were highly significant for internode volume and epidermal cell area. For pith cell length this method showed no significant difference between the normal plants and the physiological dwarfs; however, there was no significant difference between the standard deviations of these values, and Student's *t*-test is probably more valid.

*Anatomical comparisons.* The anatomical features of the internodes of normal, maleic hydrazide-stunted and physiologically dwarfed seedlings are typical of woody dicotyledons as shown by Figure 2. The internode of the physiological dwarf (Fig. 2 B, E), although similar in organization to the stem of the normal seedling (Fig. 2 A, D) is smaller, has much less pith and has cell walls which stain darker and appear thicker. These darker cell walls can be seen in the pith and cortex (Fig. 2 A, B, D, E), epidermis (Fig. 3 A, B), and xylem (Fig. 3 D, E). The similarity of cell size in the epidermis can be seen in Figure 3 A and B. The pith cells of the stems of normal seedlings (Fig. 3 G) are much larger than those of the physiological dwarf (Fig. 3 H). Representative sets of leaves from normal and physiologically dwarfed seedlings are shown in Figure 2 G.

The stems of the maleic hydrazide-stunted plants (Fig. 2 C, F) are



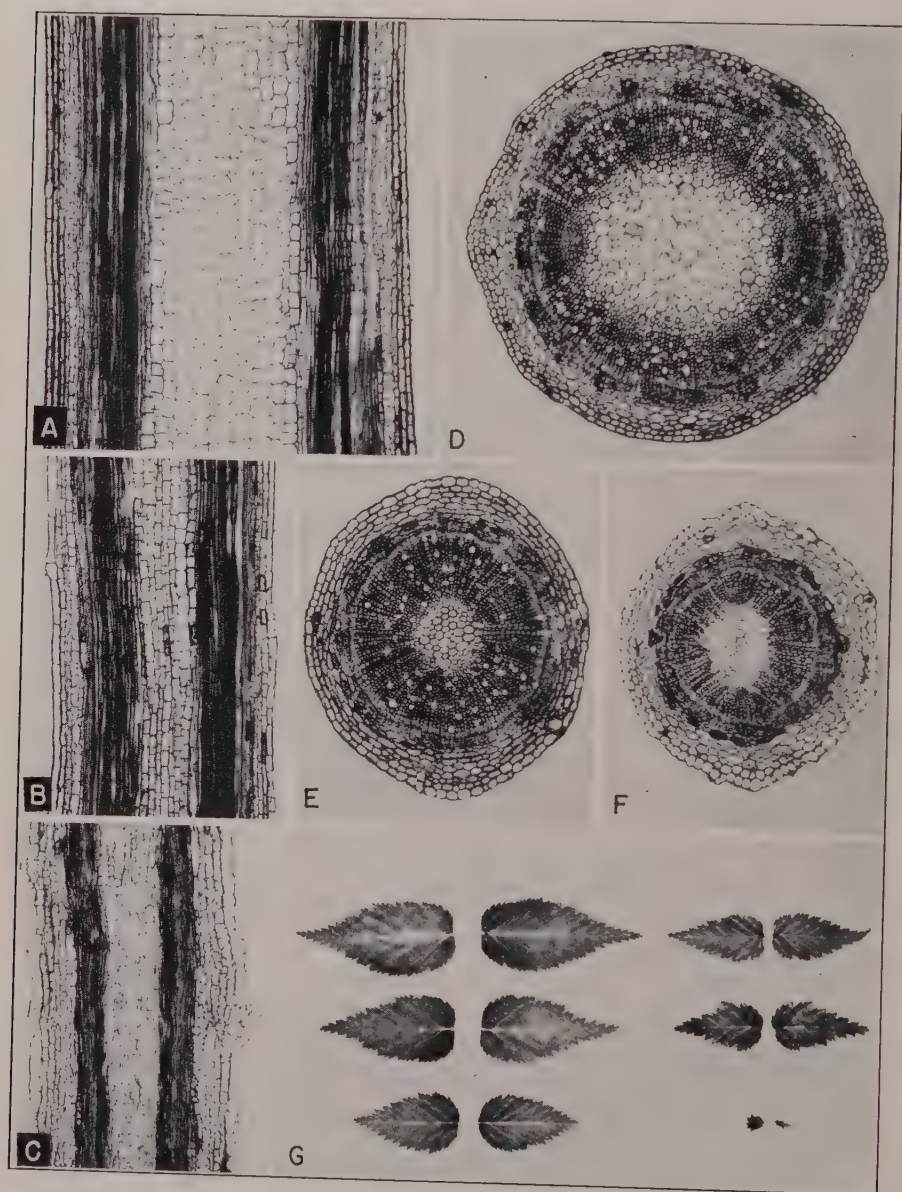


FIGURE 2. Photomicrographs of sectioned first internodes (A to F) and leaves (G) of jetbead seedlings. A, D. From normal plant. B, E. From physiological dwarf. C, F. From plant of chilled embryo treated with maleic hydrazide. G. First three pairs of leaves from normal seedlings (left) and physiologically dwarfed seedlings (right) grown five weeks in greenhouse. First formed leaves are at the bottom. (A to F,  $\times 40$ ) (G,  $\times 0.4$ ).

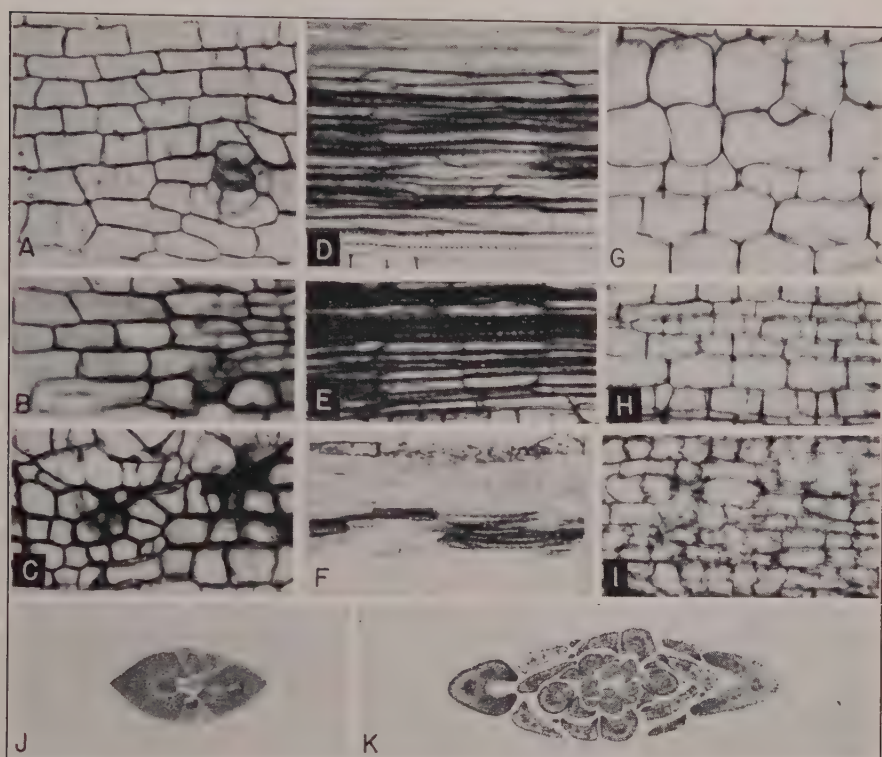


FIGURE 3. Photomicrographs of regions in sectioned first internodes of jetbead (A to I) and cross sections of peach epicotyls at the level of the shoot apex (J, K). A, B, C. Epidermis in tangential section. D, E, F. Xylem in median longitudinal section. G, H, I. Pith in median longitudinal section. A, D, G. From normal plants of chilled seed. B, E, H. From physiological dwarf. C, F, I. From plants of chilled embryos treated with maleic hydrazide. J. Epicotyl of peach early in chilling process. K. Fully after-ripened epicotyl. (A to I,  $\times 150$ ) (J, K,  $\times 17$ ).

similar to those of the physiologically dwarfed seedlings but have light-staining cell walls that appear thicker than those of either the normal or physiologically dwarfed plants. The cells of the stunted plants show an uneven pattern which lacks the strong polarity displayed by the cells of the normal plants. This can be seen in the epidermis (Fig. 3 C) where large dark areas probably represent necrotic cells and in the irregular structure of the pith (Fig. 3 I). The xylem cells (Fig. 3 F) appear to be irregularly lignified.

*Leaf development and internode length.* The highly significant correlation between the length of the first internode and the length of the first pair of leaves suggested that developing leaves have a direct and positive effect on internode growth. If the expanding leaf primordia promote internode

growth, their removal from germinating embryos should result in shorter internodes adjacent to the site of removal.

The plants from which the first pair of leaf primordia was removed showed no injury other than the lack of their first pair of leaves. Evidence that little injury was produced by this operation was found in the normal development of the stipules which were adjacent to the site of leaf removal. Only plants which were free of lateral branches were used in these measurements. When normal seedlings were compared with those from which leaf primordia were removed it was found that the first four internodes starting above the cotyledons of the normal seedlings were 2.4, 2.1, 1.5, and 0.99 times as long. Similar results were found from another lot of seeds which had been chilled 15 weeks.

Apical dominance was also affected by the removal of leaf primordia. None of the 61 control plants from the two lots of chilled seed developed lateral branches; however, 10 of the 29 plants from which the leaf primordia were removed developed one or two lateral branches at the cotyledonary node. These seedlings with lateral branches had even shorter internodes than those free of laterals.

*Changes in peach epicotyls during chilling.* Microscopic examination of epicotyls of peach seed revealed several changes that take place during the chilling process (Fig. 3 J, K). The bud expanded, cells enlarged and storage bodies within them disappeared, mitosis occurred in some cells, vascular and epidermal differentiation took place, and the stipules developed.

New leaf primordia are formed during chilling of the peach seed as shown by Figure 4. In these epicotyls the average number of leaf primordia increased from 9.5 for unchilled embryos to 10.5 after 70 days of chilling when the seed were fully after-ripened.

#### DISCUSSION

The limited extension of the first internode of the physiological dwarfs of jethead is due mostly to insufficient cell division. The cells of the epidermis and two outer layers of the cortex of the internodes of the physiological dwarfs were approximately the same size as comparable cells of the normal plants. However, the fibers, vessel elements, and especially the pith cells fail to extend normally, possibly due to insufficient turgor pressure within the cells, a greater tension in the outer tissues, especially the cambium (7), or a greater rigidity of the cell walls. It is noteworthy that the cell walls in the pith of the physiological dwarf appear thicker, and take up more stain than those of the normal seedlings. However, it is not known at what stage of development this wall thickening takes place. Recently the stems of peach plants from partially chilled seed have been investigated (33). In the stems of various heights the pith of the shorter



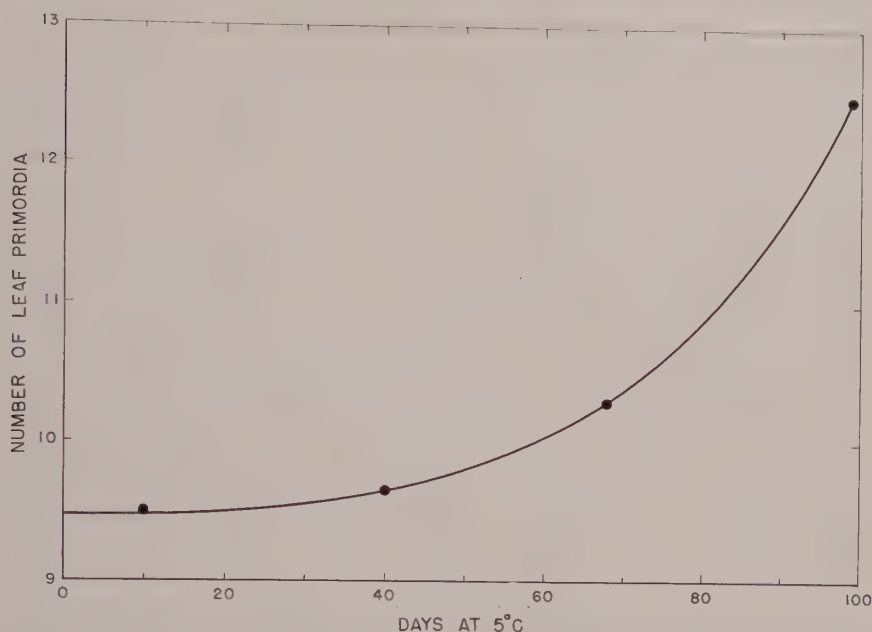


FIGURE 4. Mean leaf primordia present in epicotyls of peach seed during chilling treatment. Each point is an average of 3 or 4 determinations.

plants was predominately inhibited in cell division, and was also suppressed in cell elongation.

The simple correlations between first internode and cellular characteristics used in computing the partial correlation coefficients reported here for jetbead were similar in most respects to Sinnott's findings with *Acer* petioles (45). However, caution should be used in drawing any conclusions from these simple correlations. As Walker and Lev have pointed out (54, p. 340), "Sometimes the computed coefficient of correlation between two variables is misleading because there is little or no intrinsic correlation between them beyond what is induced by their common dependence upon a third variable (or upon several others)."

The close partial correlation between internode length and pith cell number and size in this study suggests that development of the pith may be an important factor in internode extension. This is interesting in light of the findings for *Bryophyllum* that the pith is under the greatest compression of all of the tissues of the stem (7). The pressure developed within the pith is probably an important factor in the elongation of the internodes. The limited pith development and expansion in the internodes of the physiological dwarfs evidently accounts for their failure to elongate.



This gives a significance to the pith in the early stages of internode development, even if it does not seem to have an important function later on.

The partial correlations between the shape of the internode and the shape of the cells is high for the second cortical layer and the pith. In this case the development of both of these inner tissues appears to be important in determining internode shape.

Sinnott (45) suggested that the constancy of cell size in organs of varying size reflects the degree of specialization of the cell types; however, factors which may be just as important in determining cell size are the sequence of cessation of cell division (46), and the tensional and compressional forces within the organ (7). Sinnott (46) speculates that the fundamental tissues of most axial organs have a developmental history similar to that which he describes for cucurbit fruits in which cessation of cell division takes place first in the innermost tissues and then proceeds to the successive outer layers. Once cell division ceases, subsequent organ enlargement is by cell expansion. Tissue tensions and compressions will determine, in part, the degree of enlargement which takes place. Thus, if cell division ceases first in the pith, which is under heavy compression (7), we would expect more variability in pith cell size than in the epidermal and cortical cells. In these latter types cell divisions probably persist the longest, and these tissues are under only slight compression. This may also explain the relatively constant cell size in leaves of differing size, as found in this and other studies (49), since their tissues lack the strong tensions and compressions found in the stem tissues.

Treatment of embryos from chilled seed, with maleic hydrazide, a known inhibitor of cell division (29), resulted in seedlings with even fewer cells per first internode than those of the physiological dwarfs. Therefore, most of the cells in the mature internodes of plants from chilled seed must be formed during germination and early growth of the seedling rather than during the chilling treatment.

The xylem of the maleic hydrazide-treated seedlings was not so well lignified as that of the normal or physiologically dwarfed seedlings. The use of maleic hydrazide in high concentration (over 0.5 per cent) has been found to result in thicker cell walls in treated tomato plants (34); however, the use of low concentration (less than 0.2 per cent) of this substance results in a lack of cell wall thickening (48) and the suppression of metaxylem and secondary xylem development (30). A possible explanation for these effects from low concentration is that the maleic hydrazide results in a decrease in indoleacetic acid content by increasing the enzymatic oxidation of this auxin by its oxidase (1). A close correlation has been shown between auxin production in an intact plant and the initiation of xylem cells (36).

Secondary growth was not suppressed in the treatment of jethead as it

was in the tomatoes studied by Greulach and Haesloop (30). They suggest that the increasingly greater effect of maleic hydrazide on cell enlargement as the center of the stem is approached is related to the low oxygen tension found there. A similar relationship of effect on the size of cells was found in the present studies; however, differences in stem or petiole length may be, in general, more closely correlated with size of cells in central tissues rather than exterior tissues, as has been demonstrated for jetbead and *Acer* (45).

The removal of leaf primordia from chilled seed of jetbead caused internodes to be shorter than those of intact plants at least through three internodes. Successive internodes of the experimental plants became more like the controls and appeared to recover from the leaf removal as noted by Barlow and Hancock for apple (9). They found in their experiments on apple shoots that the removal of a single young leaf resulted in an increased internode length below and a decreased internode length above the removed leaf. The main source of nutrients for growth of the first internode of jetbead must come from the cotyledons and not from the young leaves which are small and require nutrients for their own growth. The effects of leaf removal, therefore, must be mediated through factors other than nutrition, such as the effects of growth-regulating substances.

The frequent development of lateral buds in the jetbead from which leaf primordia were removed supports Barlow's contention (8) that apical dominance is resident in the developing leaves of a bud as well as in the shoot apex.

In the peach seed used in these experiments nine or more internodes were formed at the time the seed were ripe. These internodes became normal or dwarfed according to the chilling treatment, with the extent of cell division and cell enlargement determining the length. Therefore, foreshortening of the dwarf internodes and distortions of the leaves are independent of the way their tissues were formed by the shoot apex.

In the study of the changes that occur in peach seed during the chilling treatment it was noted that the epicotyl undergoes considerable expansion and development including the initiation of new leaf primordia. Arney (3) has reported that leaf primordia are also formed during winter dormancy in buds of *Fragaria*. These expanding young leaves may be the source of factors which stimulate internode extension.

The studies on biochemical changes that occur during the chilling process have been summarized by Crocker and Barton (13, pp. 161-167). While a number of changes have been noted, none has been shown to be causative in dormancy or dwarfing. Recent studies (1, 10, 16) have shown that the gibberellins can at least partially replace the chilling requirements of some seed and the buds of dormant trees, but the resulting growth usually retains some of the effects of physiological dwarfing (23, 41).

Gibberellins are effective in overcoming dwarfing in seedlings of peach and jetbead only when supplemented by intermittent light (23). Gibberellic acid has been found to initiate cell division as well as cell elongation in expanding petioles of tree peony seedlings (11). One of these substances, gibberellin A<sub>1</sub>, has been isolated from a higher plant (39). A growth inhibitor which declines in mid-February and may be associated with dormancy (31) has recently been isolated from dormant peach flower buds and identified as naringenin (32). Cotyledonary tissue exerts a strong inhibitory effect on peach seedlings grown from unchilled seed supplied with external nutrients (25). Although naringenin may not be involved in peach seed dormancy (24), the presence of promoters and inhibitors of growth in dormant tissues, as found in the dormant seed of *Fraxinus excelsior* (53), suggests that growth is controlled by a balance of these substances, and that the chilling treatment breaks dormancy by shifting the balance in favor of the growth-promoting substances either by increasing their concentration or decreasing the concentration of growth inhibitors.

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# SITE OF ACTION OF CAPTAN AND DICHLONE IN THE PATHWAY BETWEEN ACETATE AND CITRATE IN FUNGUS SPORES<sup>1</sup>

ROBERT G. OWENS AND G. BLAAK<sup>2</sup>

## SUMMARY

Conidia of *Neurospora sitophila* were found to contain substantial quantities of coenzyme A, aceto-CoA-kinase and citrogenase, which function in the synthesis of citrate from acetate. A sulfanilamide-acetyllating enzyme comparable to that found in pigeon liver or acetic kinase present in certain bacteria could not be demonstrated.

Captan and dichlone inhibited the apoenzyme in the system from pigeon liver, but neither toxicant affected the activity of aceto-CoA-kinase or citrogenase from spores. Both compounds inactivated CoA, which was shown to be the primary site of interaction in the metabolic pathway in which acetate is condensed with oxalacetate to form citrate in the spores. Inactivation of CoA by captan was accompanied by disappearance of sulfhydryl groups from CoA solutions. Inhibition was reversed by mild reducing agents and, upon reversal, the free sulfhydryl groups were liberated. This suggests that CoA is converted to the corresponding disulfide or trithiocarbonate upon reacting with captan and that the products are easily converted back to the original thiol form. Reports that histidine reverses the toxicity of captan could not be confirmed in the present experiments with conidia of *N. sitophila*.

## INTRODUCTION

Previous studies on the mode of action of dichlone (2,3-dichloro-1,4-naphthoquinone) (11) and captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] (12) showed that the toxic mechanism involves simultaneous inactivation of certain oxidative enzymes, carboxylases, and enzymes concerned in citrate synthesis and in phosphorus metabolism. Terminal oxidases apparently were not affected, since degradation of many intermediates continued through oxidative channels. Both toxicants were found to inhibit synthesis of citrate from acetate, and it was suggested that inhibition resulted from inactivation of coenzyme A. However, the possibility of inactivation of some other essential component in the pathway was not eliminated.

The present study was carried out to determine the effects of the toxicants on each step in this pathway and thus to locate the precise site of action. Since no details about the pathway in spores were available, it was first necessary to determine the normal sequence of transformations and the enzymes involved. This report provides data on these points, and

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presents further evidence that coenzyme A is the primary site of action of dichlone and captan in the pathway between citrate and acetate.

#### MATERIALS AND METHODS<sup>3</sup>

Captan and dichlone were crystallized from commercial preparations as described previously (11, 12). The samples used in these experiments melted at 171 to 172° C. and 196 to 197° C., respectively.

Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge were used in tests on intact spores and as the source of CoA, aceto-CoA-kinase, and citrogenase in some experiments. Methods of culture, harvesting, determination of dry weight, and preparation of conidia for tests were the same as described earlier (11, 12).

The sulfanilamide-acetyating enzyme from pigeon liver was prepared and assayed as described by Kaplan and Lipmann (5). CoA (75 per cent pure) and acetone-prepared liver powder for the enzyme were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. The method of Bratton and Marshall (2) was used to determine the amount of sulfanilamide acetylated during the reaction.

The effects of captan and dichlone on CoA *in vitro* were determined by preincubating each toxicant at various concentrations with 2 Kaplan and Lipmann units (5) of CoA for one hour in water. The excess toxicant was then inactivated by addition of excess cysteine-HCl and the solution was mixed with other components of the assay mixture. The complete system is described in the legend for Figure 1.

To determine the effects of the fungicides on CoA in intact spores, the spores were incubated with each toxicant at various concentrations. About 450 mg. of spores (dry weight) were suspended in 60 ml. of water in 250-ml. Erlenmeyer flasks on a reciprocating shaker. The toxicants were added in acetone and an equal amount of acetone was included in the controls. The samples were aerated by shaking for 50 minutes after addition of the toxicants and then 10 mg. of cysteine-HCl in 2 ml. of water were added to inactivate the unreacted toxicant. After 10 minutes, the samples were filtered and the spores from two flasks (about 900 mg. dry wt.) were combined and suspended in 5 ml. of distilled water in test tubes. The tubes were placed in a boiling water bath for 3.5 minutes and then cooled in an ice bath. The spores were removed by filtration and the filtrate was assayed for CoA in the same manner indicated above for authentic CoA.

The effects of the fungicides on apoenzymes were determined by the same assay procedure. However, for these experiments the toxicants were preincubated with the apoenzymes instead of with CoA for one hour.

<sup>3</sup> Abbreviations used in this paper are CoA for coenzyme A, ATP for adenosine triphosphate, and AMP for adenosine monophosphate.

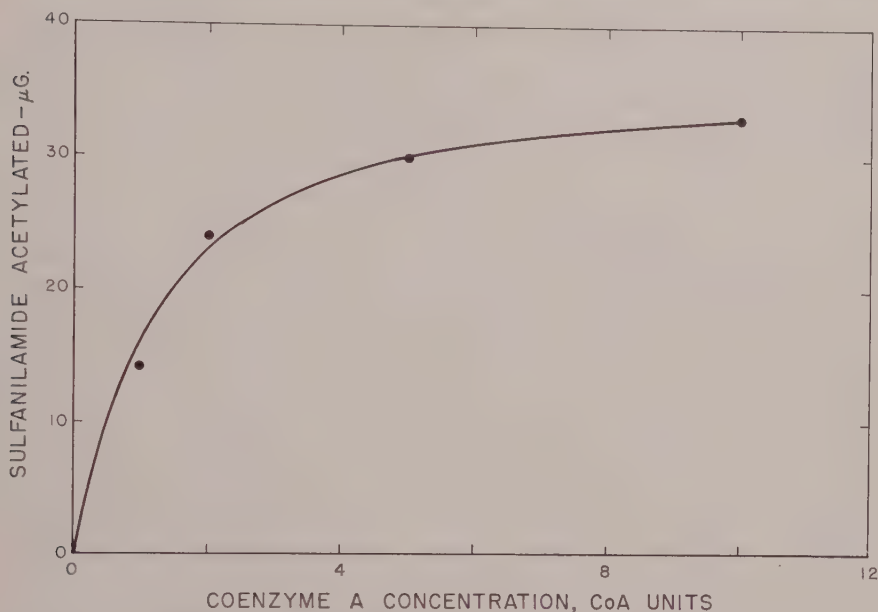


FIGURE 1. Effects of CoA at several concentrations on sulfanilamide acetylation by pigeon liver acetylase. Components of the assay mixture were as follows:  $0.39 \mu M$  of sulfanilamide;  $25.5 \mu M$  of sodium acetate;  $3.9 \mu M$  of  $\text{Na}_2\text{ATP}$ ;  $19.7 \mu M$  of sodium citrate;  $80.0 \mu M$  of  $\text{NaHCO}_3$ ;  $10 \mu M$  of cysteine hydrochloride in 0.45 ml. of water. The solution was mixed with 0.25 ml. of aged enzyme solution. CoA solution and water were then added to make a total volume of 1.4 ml. Incubation was for 2 hours in air at  $29^\circ \text{C}$ .

Excess toxicant was inactivated with cysteine or glutathione before mixing the enzyme and CoA solutions.

#### PREPARATION OF ACETO-CoA-KINASE FROM SPORES

Fifteen grams of spores (fresh wt.) were suspended in 30 ml. of  $0.1M$  phosphate buffer, pH 7.1, and treated with sonic waves for 40 minutes in the treatment cup of a Raytheon 250 w., 10 kc. magnetostrictive oscillator. Spore walls and intact spores were removed from the homogenate by centrifugation. The supernatant solution was cooled to  $3^\circ \text{C}$ . and ammonium sulfate was added with stirring until the solution was 35 per cent saturated. The precipitate was sedimented by centrifugation at  $23,500 \times g$ . for 10 minutes in a Servall centrifuge and discarded. The temperature of the supernatant solution, which had increased somewhat during centrifugation, was brought down to  $3^\circ \text{C}$ . by immersing the container in ice. Ammonium sulfate was added to 55 per cent saturation and the precipitate was collected as before and kept cold until it was used in the assay. The supernatant solution was discarded. For the assay, the precipitate was dissolved in 5 ml. of cold  $0.05M$  potassium bicarbonate buffer solution and



0.3 ml. of this containing 1.26 mg. of protein was used in each sample. Other components of the assay system are given in the legend for Figure 4. Activity was measured as described by Jones and Lipmann (4). Acetyl hydroxamate was determined by the method of Lipmann and Tuttle (6).

#### PREPARATION OF CITROGENASE FROM SPORES

Forty-five grams of spores (fresh wt.) were suspended in 100 ml. of 0.1M phosphate buffer, pH 7.1. The suspension was divided into three portions and each portion was treated for 40 minutes in the treatment cup of the sonic oscillator. Spore walls and unfragmented spores were removed by centrifugation and the supernatant solution from each portion was cooled to 3° C. After sonic treatment of the last portion, the sonates were combined and ammonium sulfate was added until the solution was 35 per cent saturated. The precipitate was removed by centrifugation at 23,500X g. and discarded. The supernatant was recooled and brought to 75 per cent saturation with ammonium sulfate. The precipitate was collected by centrifugation as above and saved. It was then dissolved in 10 ml. of cold distilled water and used for the enzyme assay. Activity was measured as described by Marcus and Elliott (9). Details of the reaction system are given in the legend for Figure 5. In the assay procedure, synthesis of citrate was stopped by immersing the assay tubes in a water bath at 90° C. for five minutes. This also destroys oxalacetate which interferes with citrate determination. The solution was cooled to room temperature and deproteinized with 0.7 ml. of 14.3 per cent trichloroacetic acid solution, followed by centrifugation. One ml. of the solution was used for each determination of citrate, as described by Saffran and Denstedt (17).

The spore homogenate was tested for acetic kinase by the method of Rose *et al.* (16) and no activity was obtained. Protein content of all enzyme preparations was determined by the modified biuret procedure described by Robinson and Hogden (14), with crystalline pepsin as the standard.

Determinations of free sulfhydryl groups in spore extracts and other solutions were made with bis(4-nitrophenyl) disulfide as described by Ellman (3).

#### RESULTS

The response of the sulfanilamide acetylating enzyme from pigeon liver to various amounts of authentic CoA is shown in Figure 1. Although more than 10 Kaplan and Lipmann units (5) of CoA were required for maximum acetylation, the increments of increase due to each unit above 2 were relatively small. Under the same assay conditions, spore extracts containing CoA produced the same response when comparable enzyme preparations were employed. This is shown in Figure 2, where authentic CoA and the spore extracts are compared. The spores consistently yielded about one CoA unit/40 to 50 mg. of spores (oven dry wt.), which was

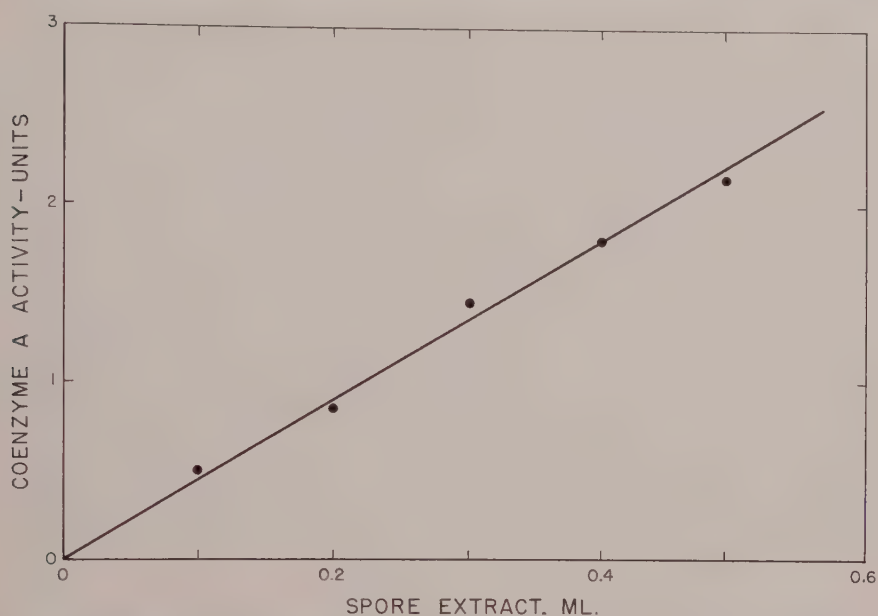


FIGURE 2. Effects of CoA extracts from *N. sitophila* spores on sulfanilamide acetylation by pigeon liver enzyme. Assay mixture is described in the legend for Figure 1.

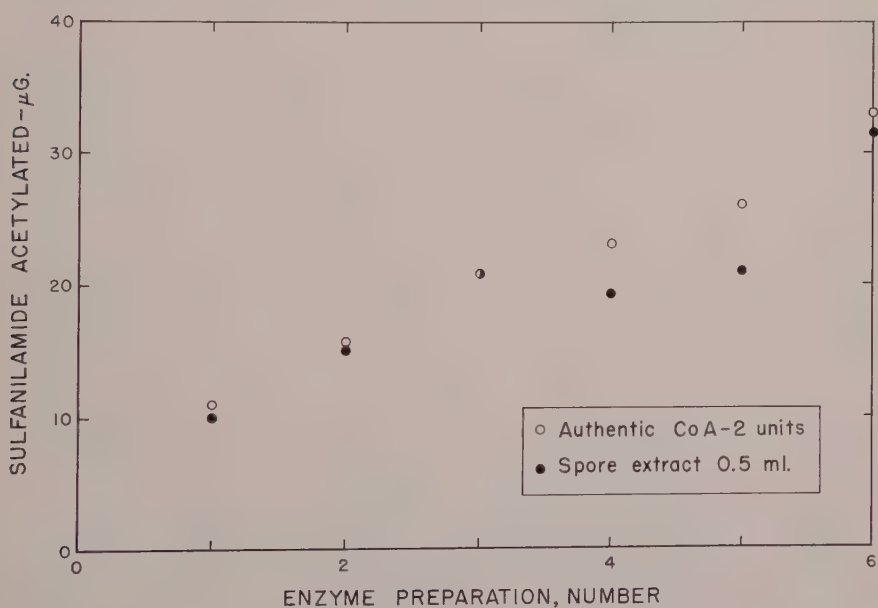


FIGURE 3. Comparative activity of authentic CoA and CoA extracts from spores of *N. sitophila* in the acetylation reaction with different preparations of pigeon liver enzyme. Assay mixture is described in the legend for Figure 1.

equivalent to slightly more than 0.2 ml. of hot water extract (Fig. 3). However, the acetylation values obtained with six different enzyme preparations from pigeon liver ranged from 12 to about 36  $\mu$ g. of sulfanilamide, even though the amount of CoA was held constant (Fig. 2).

Since these experiments indicated the presence of active CoA in the spores, several attempts were made to obtain an enzyme from the spores comparable to the sulfanilamide acetylating enzyme from pigeon liver. Spore powders prepared in a manner similar to that used for pigeon liver powders, as well as whole homogenates of spores disintegrated by sonic treatment, showed no activity. The homogenate was further tested for acetic kinase activity (16), again with negative results. These failures to obtain enzyme activity of spores comparable to that exhibited by liver suggested inadequate preparation techniques or the involvement of other enzymes in the formation of citrate from acetate by spores. The latter possibility seems more likely, for by using the methods outlined above, highly active preparations of aceto-CoA-kinase and citrogenase were obtained (Figs. 4 and 5).

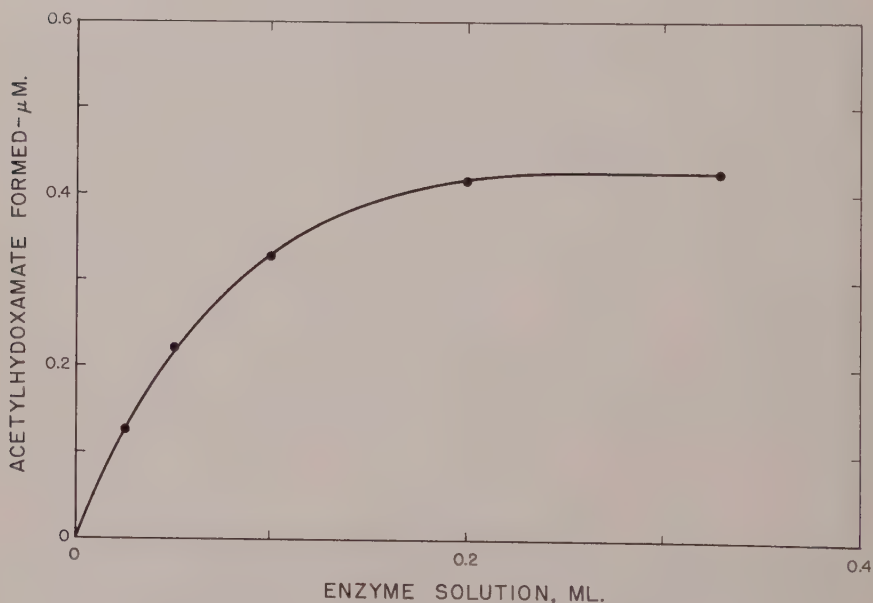


FIGURE 4. Activity of aceto-CoA-kinase preparations from *N. sitophila* spores. The assay system was as follows: 25 units of CoA; 10  $\mu$ M of  $K_2H_2ATP$ ; 20  $\mu$ M of K acetate; 50  $\mu$ M of KF; 10  $\mu$ M of  $MgCl_2$ ; 10  $\mu$ M of neutralized glutathione; 100  $\mu$ M of potassium phosphate buffer, pH 7.5; 200  $\mu$ M of  $NH_2OH$ ; enzyme solution containing 1.26 mg. of protein. Total volume, 1.0 ml.; pH, 7.5; temperature, 37° C.; incubated 20 minutes in air. The reaction was stopped by addition of 1.5 ml. of a solution containing 10 per cent  $FeCl_3 \cdot 6H_2O$ , 3.3 per cent trichloroacetic acid, and 0.66N HCl. Color density read at 540  $m\mu$  and compared with blank containing 1 ml. of water and 1.5 ml. of the above reagent.

## EFFECTS OF DICHLONE AND CAPTAN ON THE ACTIVITY OF CoA

Although the experiments reported above suggested the absence of a sulfanilamide acetylating system in spores, pigeon liver acetylase was used to determine the effects of dichlone and captan on CoA activity because it was readily available and has a specific requirement for CoA. Each toxicant was incubated for one hour with CoA in water prior to adding the CoA solution to other components of the assay system. Unreacted toxicant was destroyed by addition of excess cysteine at the end of the preincubation period. All components of the assay mixture were then combined, initiating the acetylation reaction. The results given in Figure 6 show that dichlone strongly inhibited CoA activity whereas captan was not very inhibitory even at a concentration of 100  $\mu\text{g./ml.}$  of solution.

Similar results were obtained in studies on the effects of dichlone and captan on CoA in intact spores. The spores were exposed for about 50 minutes to several dosages of the toxicants, and then the unreacted fungicide was inactivated with excess cysteine. The remaining CoA was extracted with hot water and tested for activity. The results are given in Figures 7 and 8 along with data on germination. CoA activity and ger-

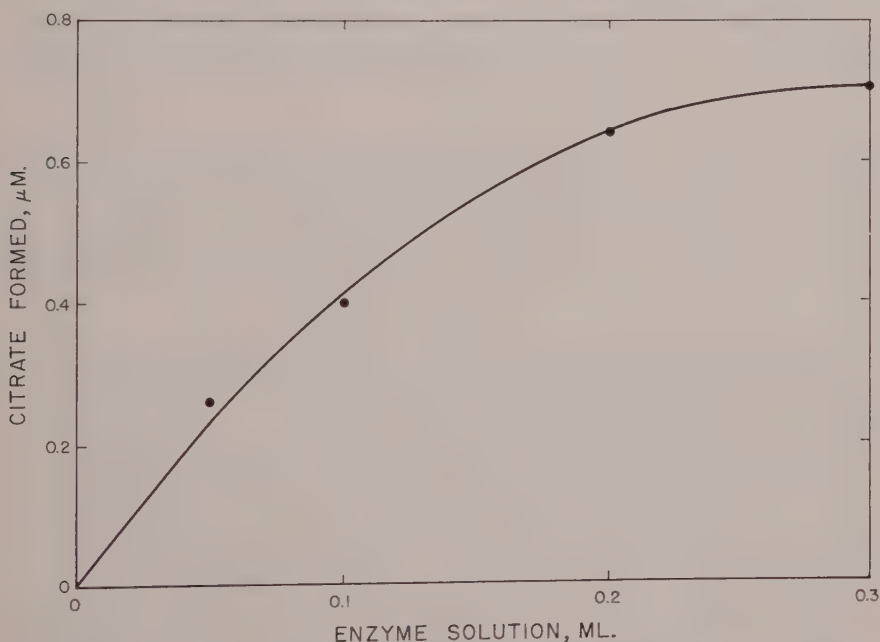


FIGURE 5. Activity of citrogenase preparations from *N. sitophila* spores. The assay system was as follows: 10 units of CoA; 10  $\mu\text{M}$  of neutralized glutathione; 10  $\mu\text{M}$  of  $\text{MgCl}_2$ ; 100  $\mu\text{M}$  of K acetate; 15  $\mu\text{M}$  of  $\text{K}_2\text{H}_2\text{ATP}$ ; 25  $\mu\text{M}$  of K oxalacetate; 150  $\mu\text{M}$  of potassium phosphate buffer, pH 7.5; enzyme solution containing 3.76 mg. of protein. Total volume, 1.25 ml.; pH, 7.5; incubated 90 minutes at 29° C. in air.



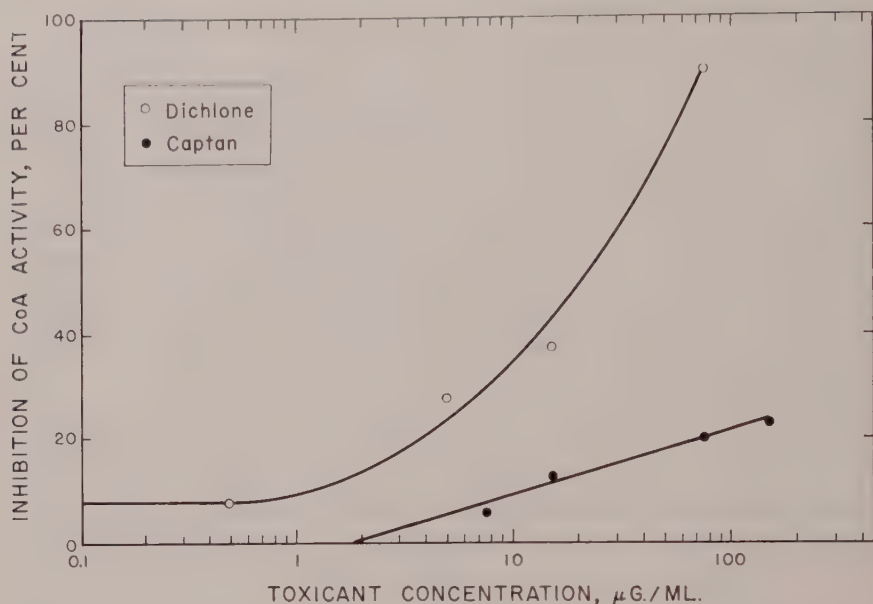


FIGURE 6. Effects of dichlone and captan on CoA *in vitro*. The toxicants were preincubated with 2 units of CoA for one hour and then the excess was destroyed with excess cysteine HCl prior to mixing with other components of the assay system. Assay mixture described in the legend for Figure 1.

mination were inhibited by dichlone within the same range of doses, which indicates concomitance of effects on the two functions. However, the curves do not have the same profile. This suggests that the germination curve was influenced by inhibition of other enzymes and/or coenzymes as well as CoA.

The effects of captan on germination appeared to be unrelated to effects on CoA (Fig. 8). Inactivation of CoA plotted against captan concentration yielded a curve with an inversion in all of six independent experiments. The cause of the inversion is discussed in a subsequent section.

The apparent lack of relationship between inactivation of CoA and inhibition of germination by captan was not consistent with previous studies which showed that synthesis of citrate from acetate was inhibited in intact spores (12). The possibility that the toxicants might affect apoenzymes instead of CoA was then investigated with the results given in Table I and Figure 9. The data show that neither dichlone nor captan had any appreciable effect on aceto-CoA-kinase or citrogenase from spores, although both fungicides inhibited the pigeon liver enzyme.

Since captan did not inactivate aceto-CoA-kinase or citrogenase *per se*, its effects on CoA were reexamined with special attention to the inversion

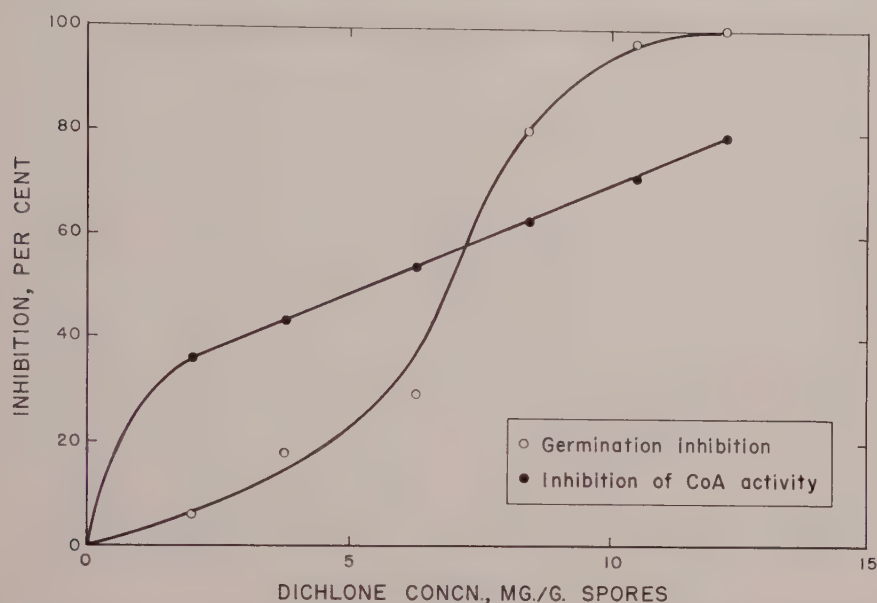


FIGURE 7. Effects of dichlone on CoA *in vivo* and on germination of *N. sitophila* spores. Assay mixture is described in legend for Figure 1, with spore extract being used instead of authentic CoA solution. Spores were pretreated with dichlone for 45 minutes before CoA was extracted.

in the inhibition curve (Fig. 8). If the initial inactivation of CoA by captan were extended in a straight line from the origin, as indicated by the broken line in Figure 8, it would coincide with inhibition of germination with respect to captan concentration. This relationship might be expected in view

TABLE I

COMPARATIVE EFFECTS OF DICHLONE AND CAPTAN ON ACETO-CoA-KINASE, CITROGENASE FROM SPORES AND THE ACETYLATED ENZYME FROM PIGEON LIVER *in vitro*\*

| Enzyme           | Toxicant | Amount of toxicant, $\mu\text{g.}/\text{mg. protein}$ | Inhibition, % |
|------------------|----------|-------------------------------------------------------|---------------|
| Aceto-CoA-kinase | Dichlone | 2.2                                                   | 3             |
|                  | Captan   | 3.2                                                   | —5            |
| Citrogenase      | Dichlone | 0.56                                                  | 8             |
|                  | Captan   | 0.80                                                  | 28            |
| Liver enzyme     | Dichlone | 0.25                                                  | 62            |
|                  | Captan   | 0.25                                                  | 43            |

\* Assay systems are described in the legends for Figures 1, 4, and 5. The toxicants were preincubated for 1 hr. with each enzyme before addition of CoA which initiates the reactions. Immediately before addition of CoA, excess glutathione was added to destroy any unreacted toxicant to prevent its reaction with the CoA.

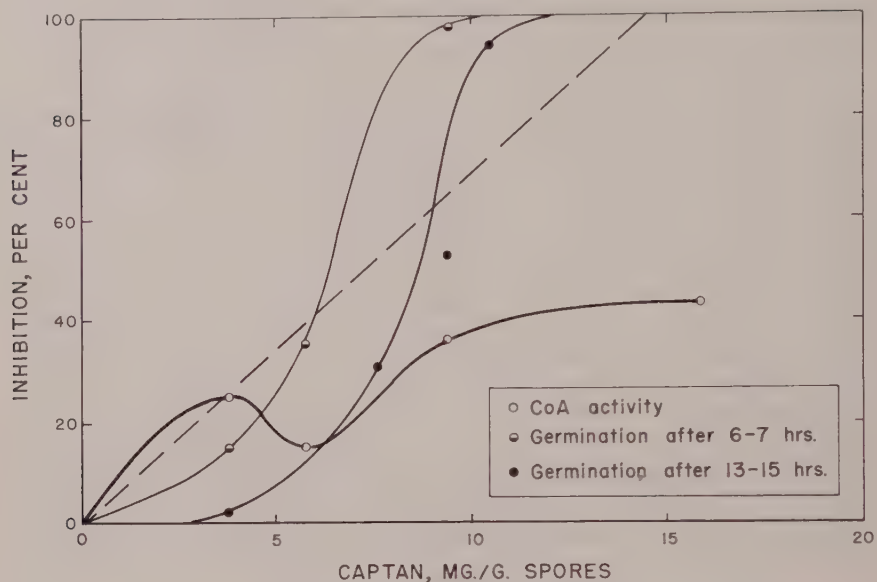


FIGURE 8. Effects of captan on CoA *in vivo* and on germination of *N. sitophila* spores. Assay mixture is described in the legend for Figure 1, with spore extract being used instead of authentic CoA solution. Spores were pretreated with captan for 45 minutes before CoA was extracted.

of the linear effect produced by dichlone above 2 mg./g. of spores (Fig. 7). The inversion suggests, therefore, that the effects of captan on CoA might have been reversed upon transferring the CoA-captan reaction products to the assay system which contained an excess of cysteine. This possibility was also suggested by the fact that reaction of captan with thiols results in the formation of disulfides (7, 10) and in some instances trithiocarbonates (10), both of which undergo exchange reactions with thiols to yield the original thiol in equilibrium with the added thiols (10).

To obtain more direct evidence on this point, spores incubated with lethal doses of captan were extracted with hot water as in previous experiments and the free sulfhydryl content of the extract was determined. It was found that 6.4 and 8.7 mg. of captan/g. of spores reduced the free sulfhydryl content by 70 and 81 per cent, respectively. Although CoA was not the only sulfhydryl compound in the extract, the data were taken as tentative evidence that a large portion of the endogenous CoA reacted with captan in intact spores. This was strengthened by further experiments *in vitro*, in which it was found that essentially all of the sulfhydryl groups of CoA disappeared within 30 minutes upon addition of captan to CoA solutions (Table II). The reversibility of the reaction, which accounts for the failure of captan to inactivate CoA in the presence of cysteine in the

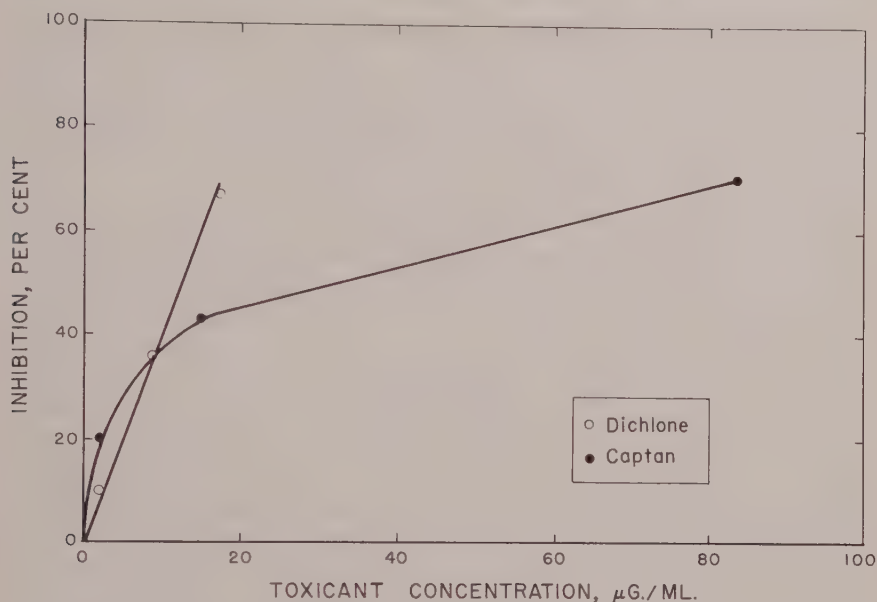


FIGURE 9. Effects of dichlone and captan on apoenzymes in pigeon liver extract. The toxicants were preincubated with the liver extract for one hour and then destroyed with an excess of cysteine HCl prior to mixing with other components of the assay system. Assay mixture as described in the legend for Figure 1.

acetylation reaction, was established by addition of thiophenol to CoA and glutathione solutions after treatment with captan. The excess thiophenol and, presumably, the corresponding disulfide resulting from its reaction with glutathione and CoA disulfides, were extracted quantitatively with ether and the free sulphydryl content of the aqueous phase was de-

TABLE II  
REACTION OF CAPTAN WITH FREE THIOL GROUPS OF CoA AND GLUTATHIONE *in vitro*  
AND REVERSAL OF THE REACTION BY THIOPHENOL

| Thiol       | Initial concentration, $\mu M$ | Captan added, $\mu M$ | Residual thiol concentration, $\mu M$ | Thiophenol added, mg.* | Residual thiol concentration, $\mu M$ | Percentage recovery |
|-------------|--------------------------------|-----------------------|---------------------------------------|------------------------|---------------------------------------|---------------------|
| Coenzyme A  | 6.05                           | 0                     | 6.05                                  | 500                    | 4.0                                   | 66                  |
| Coenzyme A  | 6.05                           | 3.02                  | 0                                     | 500                    | 4.0                                   | 66                  |
| Glutathione | 130                            | 0                     | 130                                   | 500                    | 117                                   | 90                  |
| Glutathione | 130                            | 67                    | 0                                     | 500                    | 112                                   | 86                  |

\* Thiophenol was removed from the solution quantitatively by extraction with ether before determination of the residual thiol concentration. Some CoA and glutathione were also removed as evidenced by the percentage recovery in the last column. However, the data show that the thiol concentrations after extraction of controls and treated solutions were essentially equivalent.



terminated. The data show that both glutathione and CoA were quantitatively restored to the free thiol form. This, therefore, explains the anomaly between the effects of captan on CoA in intact spores and on CoA in the *in vitro* assay system which contains excess cysteine, since cysteine would reverse the reaction as effectively as did thiophenol.

#### EFFECTS OF HISTIDINE ON TOXICITY OF CAPTAN

It has been reported that histidine, as well as thiols, reverses the toxicity of captan to certain fungi (13). Since such reversal would add a new and unexplained dimension to the problem of the mode of action of captan, several tests were carried out to examine the effects of histidine on poisoning of *N. sitophila* spores by captan. Two kinds of tests were conducted. The first was the conventional slide-germination test (1), in which spores were exposed to a lethal dose of 2 p.p.m. of captan overnight in aqueous solution. The following morning the spores were mixed with an equal volume of solutions containing 2 to 200 p.p.m. of *l*-histidine. The germination test was set up and counts were made when the controls were fully germinated. A final captan concentration of 1 p.p.m. was found to be sufficient to inhibit germination completely, and no reversal in the histidine treatments was observed even after 24 hours.

A second experiment utilizing a shorter exposure to captan was then carried out. The spores were incubated in water on a reciprocating shaker for 45 minutes with 6.2 mg. of captan/g. dry wt. of spores. Afterwards, the spores, washed to remove the excess captan, were suspended in water with

TABLE III  
EFFECTS OF HISTIDINE ON TOXICITY OF CAPTAN TO SPORES OF *NEUROSPORA*  
*SITOPHILA* AND ON FREE SULFHYDRYL CONTENT OF THE SPORES

| Treatment of conidia   |                          |                                    | Germination, % |                             |                                | Thiol content of spores, %† |
|------------------------|--------------------------|------------------------------------|----------------|-----------------------------|--------------------------------|-----------------------------|
| Captan, mg./g. spores* | Histidine, mg./g. spores | Exposure time to histidine, min.** | Control        | After treatment with captan | After treatment with histidine |                             |
| 0                      | 0                        | 0                                  | 95             | —                           | —                              | 100                         |
| 0                      | 4.1                      | 20                                 | 90             | —                           | 93                             | 96                          |
| 0                      | 8.2                      | 20                                 | 96             | —                           | 94                             | 96                          |
| 6.2                    | 4.1                      | 20                                 | —              | 0                           | 3                              | 53                          |
| 6.2                    | 8.2                      | 20                                 | —              | 0                           | 3                              | 60                          |
| 6.2                    | 0                        | 0                                  | —              | 0                           | —                              | 58                          |
| 6.2                    | 4.1                      | 40                                 | —              | 0                           | 4                              | 55                          |
| 6.2                    | 8.2                      | 40                                 | —              | 0                           | 3                              | 49                          |

\* Exposure to captan was 45 minutes.

\*\* Spores were exposed twice to histidine for the indicated time.

† Determined in hot water extracts of spores after treatment with captan and/or histidine. Percentage is based on total water soluble thiols in untreated controls.

histidine in the amounts and for the time periods indicated in Table III. Some of the spores were then tested for germination. The remainder were filtered, washed, resuspended in 5 ml. of water, and transferred to 15-ml. test tubes immersed for 5 minutes in a boiling water bath to extract the water-soluble thiols. Subsequently, the free thiol groups were determined on 0.5 ml. of the extract as indicated in the section above on Materials and Methods. The results of histidine treatment on germination and on free thiol groups are given in Table III. The data show that there was little or no tendency toward liberation of thiol groups by histidine or of reversing inhibition of germination.

#### DISCUSSION

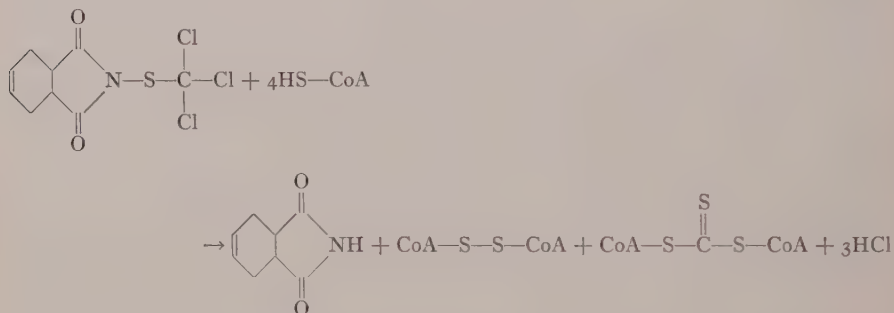
Evidence is presented that CoA, aceto-CoA-kinase, and citrogenase are present in spores of *N. sitophila*. This confirms the previous assumption that this or a similar system of enzymes is functional in spores. A sulfanilamide-acetylating enzyme comparable to that in pigeon liver and acetic kinase, such as is found in certain bacteria, could not be demonstrated. It seems likely, therefore, that the principal synthesis of citrate from acetate in the spores proceeds in three steps as follows:



This pathway is the same as that shown to dominate in yeast, higher plants, animals, and certain bacteria (4). It differs from that in bacteria such as *Escherichia coli*, *Proteus vulgaris*, *Clostridium* sp., *Streptococcus* sp. (15) in that the latter proceeds by formation of acetylphosphate in the initial step with ADP as a byproduct.

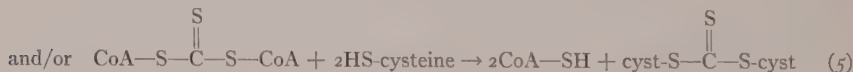
Since captan or dichlone does not inhibit the apoenzymes responsible for citrate synthesis from acetate, inhibition of citrate synthesis in intact spores, which was demonstrated previously and correlated with loss of spore viability (11, 12), must be due to inactivation of CoA. Data presented in this paper show that dichlone inactivates CoA irreversibly within the toxic range of doses. Evidence is presented that captan also reacts with CoA, but that the product formed can be reconverted to the thiol form by mild reducing agents, such as thiophenol and cysteine. The anomaly between previous data showing inhibition of citrate synthesis by captan and the present data showing the lack of inhibition by captan *in vitro* apparently is due to reversal of the captan effect by cysteine in the *in vitro* assay system.

These conclusions are in accord with the findings of Lukens and Sisler (8) who showed that captan is detoxified by CoA, indicating a reaction between the two compounds. Lukens (7) has recently shown that reaction of captan with sodium dimethyldithiocarbamate, ferbam and ziram yields tetrahydrophthalimide, tetramethylthiuram disulfide, tetramethylthiuram monosulfide, and carbon disulfide. These kinds of reaction products have been confirmed and extended by the present authors (10) to other thiols, including CoA. In some cases the thiols give rise to stable trithiocarbonates instead of monosulfides and CS<sub>2</sub>. The disulfides and trithiocarbonates are converted back to the original thiols by compounds such as glutathione and cysteine, which may reduce the disulfide linkage and/or exchange with one of the thiol anions upon dissociation of the disulfide. The reaction of captan with CoA can be written as follows:

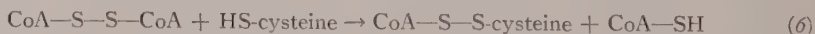


This is based on the reactions elucidated by Lukens (7) and by the present authors in another paper (10).

The reversal of the reaction, which is shown above to occur in the presence of cysteine and other thiols, probably proceeds as follows:



Hybrid products are also probable if the CoA/cysteine ratio is appropriate:

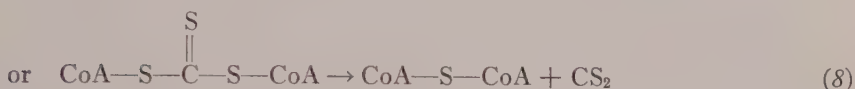
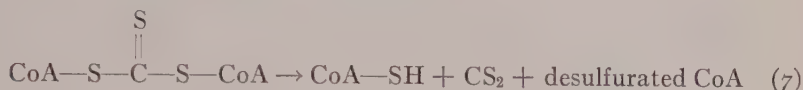


Glutathione and other thiols apparently are as effective as cysteine in the reversal reaction.

These reactions are in accord with previous studies on the action of captan on respiration and germination of *N. sitophila* spores (12), which showed that both respiration and germination tended to recover when captan was not in excess. The present data suggest that recovery is re-

lated to the internal reduction of disulfides and/or trithiocarbonates formed in the reaction of captan with vital sulfhydryl constituents of the cells.

Incomplete recovery of spore viability (12) suggests that a portion of the reactants in cells is permanently inactivated. This can be explained, in part at least, by spontaneous decomposition of some of the captan-thiol products in such a manner as to split off the sulfur atom from the original thiol. Lukens (7) has provided direct evidence that such decomposition occurs in certain cases. With CoA as an example, the decomposition of the trithiocarbonate can be visualized as follows:



In general, the same conclusions may be drawn regarding the mechanism of action of captan and dichlone that were suggested from previous studies (11, 12). Toxicity apparently results from inactivation of many enzymes and coenzymes, especially CoA, simultaneously. CoA and other thiols, such as glutathione and sulfhydryl enzymes, appear to be very important in the mechanism of action of these fungicides, and since these and other thiols are functional in many areas of metabolism, the fungicides obviously affect metabolism in all major areas (11, 12).

Reversal of captan toxicity by histidine, as reported by Rich (13), could not be confirmed with *N. sitophila* spores. Reversal in other fungi and other types of organisms remains a possibility, of course, but the mechanism of reversal would be difficult to explain in the light of present knowledge of the mechanism of captan action. Histidine had no apparent tendency to liberate sulfhydryl groups from the CoA-captan reaction product, as did cysteine and other thiols.

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# CHEMISTRY OF THE REACTIONS OF DICHLONE AND CAPTAN WITH THIOLS<sup>1</sup>

ROBERT G. OWENS AND G. BLAAK<sup>2</sup>

## SUMMARY

Dichlone (2,3-dichloro-1,4-naphthoquinone) was found to react with thiophenol, 4-nitrothiophenol, and *n*-octyl mercaptan to yield 2,3-disubstituted mercaptoquinones. Reaction with glutathione and coenzyme A apparently was not so complete as with the simpler thiols and a mixture of mono- and disubstituted products was indicated.

Reaction of captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] with thiols was more complicated. Captan was partially decomposed to tetrahydrophthalimide, hydrochloric acid, and thiophosgene. The subsequent fate of thiophosgene depended upon the nature of the thiol participating in the reaction. Thiophenol and 4-nitrothiophenol reacted with thiophosgene as rapidly as it was formed, yielding bis(phenyl) trithiocarbonate and bis(4-nitrophenyl) trithiocarbonate. Glutathione, however, reacted more slowly with thiophosgene. Consequently, thiophosgene vapor could be detected by organoleptic test and by trapping with 4-nitrothiophenol. Moreover, glutathione yielded only one detectable product, oxidized glutathione, upon reacting with captan. If the trithiocarbonate formed, it apparently decomposed in such a manner as to yield oxidized glutathione.

The data are discussed with regard to the role of thiophosgene in the fungitoxic mechanism of captan and with regard to the chemical mechanism of thiol—captan interaction.

## INTRODUCTION

Dichlone (2,3-dichloro-1,4-naphthoquinone) reacts readily with thiols and certain amines under physiological conditions of pH and temperature, and some evidence has been advanced that reaction with free sulfhydryl and amino groups of enzymes and coenzymes accounts for its toxicity to fungi (9, 10, 11, 12).

Captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] also has been shown to inactivate enzymes and coenzymes with functional sulfhydryl groups (2, 5, 7, 8, 11, 13, 16) but it apparently does not react rapidly with amino acids or peptides that lack sulfhydryl groups (2). The chemistry of the reaction with several thiols has been investigated previously. Lukens (7) found that reaction with sodium dimethyldithiocarbamate yields tetramethylthiuram disulfide, tetramethylthiuram monosulfide, tetrahydrophthalimide, carbon disulfide and sodium chloride. Lukens and Sisler (8) showed that reaction with cysteine yields a cyclic compound, 2-thiazolidinethione-4-carboxylic acid, as one of several

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products. Thiophosgene is also formed. The present authors showed that reaction with coenzyme A results in disappearance of sulfhydryl groups from CoA solutions and in simultaneous loss of the catalytic activity of CoA (11). However, CoA was restored to the thiol form by incubation with cysteine or thiophenol and it was fully reactivated. The evidence suggests, therefore, that reaction of captan with thiols involves more than a simple substitution of thiols for chlorine on the methyl carbon of captan, since substitution alone would not account for the products obtained.

The present study was carried out to examine further the details of the chemistry of the reactions of dichlone and captan with thiols. The data show that previous concepts of the reactions were incomplete with respect to certain details, and they indicate that the structure of the thiol has a strong influence on the extent to which the reactions proceed.

#### MATERIALS AND METHODS

Dichlone and captan with melting points of 196–197° C. and 171–172° C., respectively, were used. Each was purified from commercial fungicide preparations as described previously (12, 13). 4-Nitrothiophenol was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; thiophosgene from Rapter Laboratories, Chicago, Illinois; and all other special reagents from Distillation Products Division of Eastman Kodak Company, Rochester, New York.

Free sulfhydryl groups were assayed with bis(4-nitrophenyl) disulfide as described by Ellman (3). Chloride was determined with silver diphenylthiocarbazone as described by Kirsten (6). Infrared curves were obtained with a Perkin-Elmer spectrophotometer, Model 221. Ultraviolet spectra were determined with a Perkin-Elmer automatic recording spectrophotometer, Model 4000 A. Elemental analyses were made by Schwartzkopf Microanalytical Laboratory, New York, New York.

Details of reaction conditions and isolation procedures are given in the text since they differed somewhat for each thiol.

#### RESULTS

##### REACTION OF DICHLONE WITH THIOLS

The 4-nitrothiophenate ion is yellow in aqueous solution and the color intensity increases as the pH is increased, up to the pH at which ionization is complete. Acylation or arylation of the compound diminishes the color intensity in proportion to the amount reacted. Therefore, it was possible to follow the reaction of dichlone or captan with 4-nitrothiophenol to the end point simply by adding the thiol in an organic solvent to a solution of either fungicide until the yellow color just persisted, or, conversely, by adding the fungicide to the thiol until the color just disappeared. In the case of dichlone the titration had to be carried out to a constant color

density because the reaction product absorbed at about the same wavelength as the 4-nitrothiophenolate ion. However, in practice this was not a serious handicap because the thiol anion had a greater extinction coefficient than the reaction product and the end point was revealed by a change in slope of the titration curve.

*Reaction of dichlone with 4-nitrothiophenol.* Because of the convenience afforded by visible evidence of the end point in the reaction, 4-nitrothiophenol was used to determine the amount of thiol with which dichlone was capable of reacting. When the reaction was complete, the relationship between thiol consumed and chloride liberated was determined as an indication of the extent of substitution and of the possible product. Data in Table I show that two moles of 4-nitrothiophenol reacted for each mole of

TABLE I  
MOLAR RELATIONSHIPS BETWEEN THE AMOUNTS OF 4-NITROTHIOPHENOL CAPABLE OF REACTING WITH DICHLONE AND CAPTAN AND THE AMOUNTS OF CHLORIDE LIBERATED DURING THE REACTIONS\*

| Toxicant | Micromoles     |       |                   | Ratios         |             |          |
|----------|----------------|-------|-------------------|----------------|-------------|----------|
|          | Amount reacted |       | Chloride released | Thiol/toxicant | Cl/toxicant | Cl/thiol |
|          | Toxicant       | Thiol |                   |                |             |          |
| Dichlone | 15.0           | 29.0  | 27.0              | 1.93           | 1.80        | 0.93     |
| Captan   | 10.0           | 38.5  | 29.3              | 3.85           | 2.93        | 0.76     |

\* The toxicants were dissolved in acetone. 4-Nitrothiophenol was dissolved in 90 per cent aqueous acetone solution containing 1 gram of sodium acetate as a partial buffer. The thiol was added dropwise to the toxicant solution until the yellow color of the 4-nitrothiophenol anion just persisted. The amount of thiol added was then calculated on the basis of volume. Chloride analyses were made when the titrations were complete.

dichlone present in the reaction system. Two chlorine atoms were released, indicating that dichlone was substituted at both the 2 and 3 positions. Substitution at both positions was confirmed in a subsequent experiment in which the product was isolated and characterized by elemental analysis. The reaction mixture for this experiment consisted of 136 mg. of dichlone in 200 ml. of absolute ethanol and 160 mg. of 4-nitrothiophenol in 10 ml. of ethanol. Forty ml. of 1 per cent aqueous sodium acetate solution were added to prevent a drastic drop in pH. The reactants were incubated for 30 minutes at room temperature and then the solution was cooled in a dry ice bath and filtered. The precipitate was dissolved in a minimum of a mixture of ethanol and acetone, 1:2 v/v. The volume of the solvents was reduced by evaporation and orange crystals formed, which, upon recrystallization, melted at 235° C.

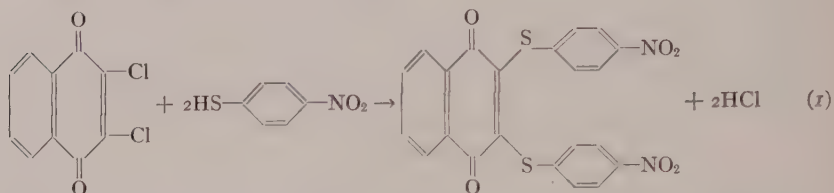
*Anal.* Calcd. for the disubstituted product,  $C_{22}H_{12}N_2O_6S_2$ : C, 57.0; H, 2.6; N, 6.0; O, 20.6; S, 13.8. Found: C, 59.1; H, 3.2; N, 6.8; O, 14.7; S, 13.7.



The O analysis was somewhat low but values for all other elements agree with those calculated for 2,3-di-(4-nitrophenylmercapto)-1,4-naphthoquinone.

The ultraviolet absorption spectrum of the derivative is shown in Figure 1 A. Infrared absorption characteristics are shown in Figure 2 A. The peak at a frequency of  $1670\text{ cm}^{-1}$  was produced by carbonyl groups and it shows that the quinone was not reduced during the reaction.

The reaction can be written as follows:



*Reaction of dichlone with thiophenol.* Thiophenol, 49.0 mg., was mixed with 50 mg. of dichlone in absolute ethanol. Other conditions and procedures were the same as described for 4-nitrothiophenol. The recrystallized product melted at  $144\text{--}145^\circ\text{C}$ .

*Anal.* Calcd. for the disubstituted quinone,  $\text{C}_{22}\text{H}_{14}\text{O}_2\text{S}_2$ : C, 70.6; H, 3.7; O, 8.6; S, 17.1. Found: C, 71.0; H, 4.1; O, 8.6; S, 16.9; Cl, 0.2. These values agree with those calculated for the disubstituted product, 2,3-di-(phenylmercapto)-1,4-naphthoquinone.

The ultraviolet absorption spectrum of the compound is given in Figure 1 B. Infrared absorption characteristics are shown in Figure 2 B. The curve again shows the carbonyl peak at a frequency of  $1670\text{ cm}^{-1}$ , indicating that the quinone was not reduced during the reaction.

The reaction may be written as shown above for 4-nitrothiophenol.

*Reaction of dichlone with *n*-octyl mercaptan.* *N*-Octyl mercaptan, 65 mg. in about 10 ml. of absolute ethanol, was mixed with 50 mg. of dichlone in 70 ml. of ethanol. Sodium acetate, 100 mg. in 10 ml. of water, was added to prevent a drastic drop in pH. Upon standing, needle-like crystals precipitated, m.p.  $58\text{--}59^\circ\text{C}$ .

*Anal.* Calcd. for the disubstituted quinone,  $\text{C}_{26}\text{H}_{38}\text{O}_2\text{S}_2$ : C, 70.0; H, 8.5; O, 7.2; S, 14.3. Found: C, 69.2; H, 9.1; O, 8.3; S, 14.2. The value for O was slightly high but values for other elements agree with those calculated for 2,3-di-(*n*-octylmercapto)-1,4-naphthoquinone.

The ultraviolet absorption spectrum of the compound is shown in Figure 1 C. The infrared absorption spectrum is shown in Figure 2 C. The curve shows that the carbonyl peak shifted slightly to a frequency of  $1650\text{ cm}^{-1}$  indicating that the quinone was not reduced. The peaks at frequencies of  $2825$  and  $2900\text{ cm}^{-1}$  are due to the hydrocarbon chains.

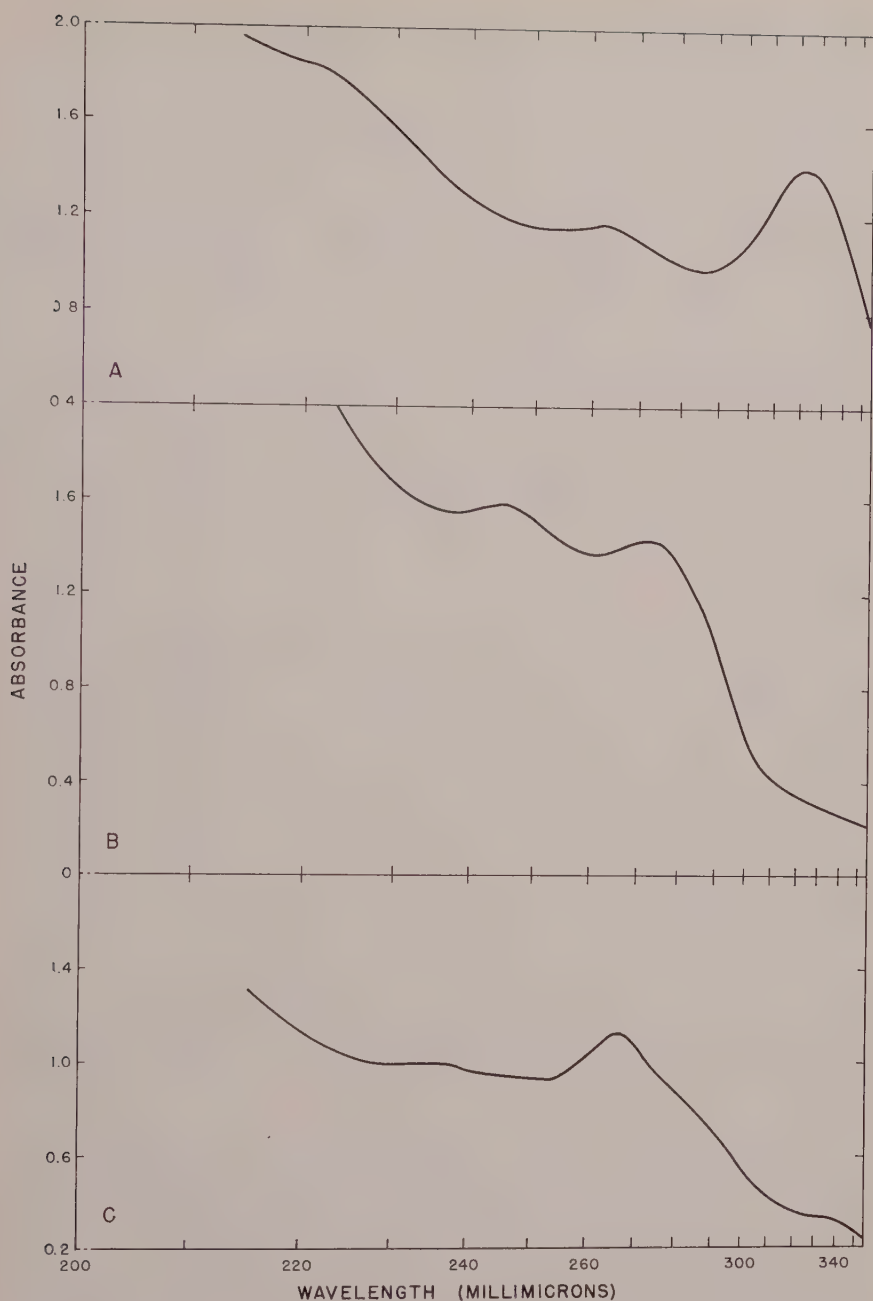


FIGURE 1. Ultraviolet absorption spectra of: (A) 2,3-di-(4-nitrophenylmercapto)-1,4-naphthoquinone derived from reaction of dichlone with 4-nitrothiophenol (concentration, 30  $\mu\text{g.}/\text{ml.}$ ), (B) 2,3-di-(phenylmercapto)-1,4-naphthoquinone derived from reaction of dichlone with thiophenol (concentration, 25  $\mu\text{g.}/\text{ml.}$ ), and (C) 2,3-di-(*n*-octylmercapto)-1,4-naphthoquinone derived from reaction of dichlone with *n*-octylmercaptan. Solvent, absolute ethanol.

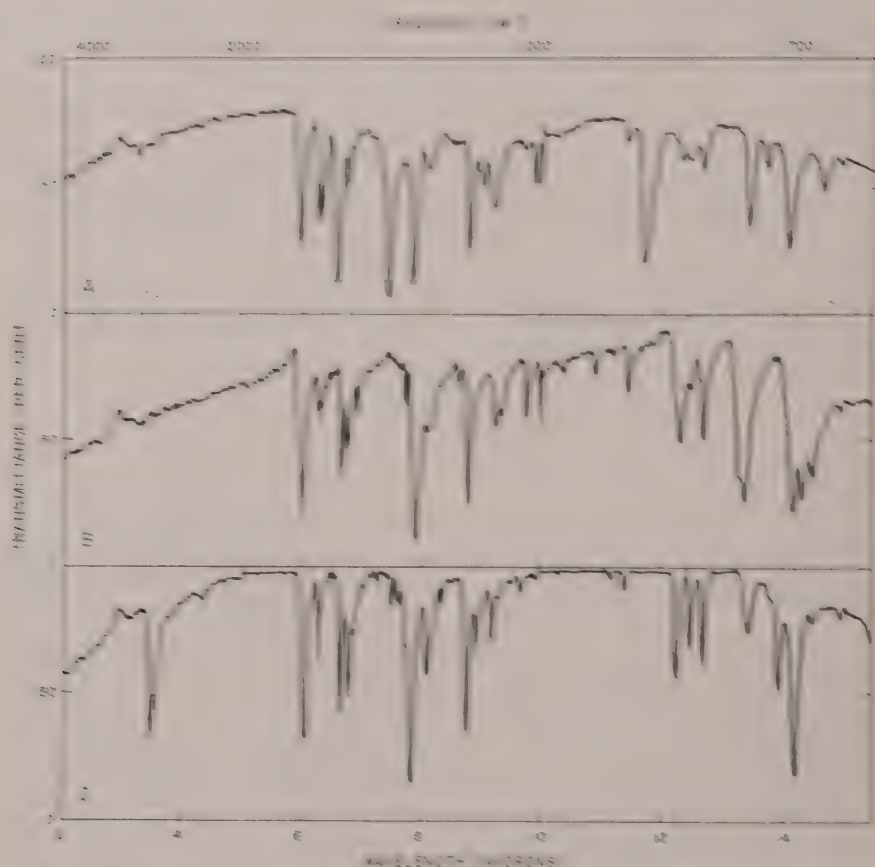


FIGURE 1. Infrared absorption spectra of: (A) 2,3-di-*n*-octylthiopyran-1,4-naphthoquinone derived from reaction of dichlorone with 2-nitrothiophenol; (B) 2,3-di-*n*-octylthiopyran-1,4-naphthoquinone derived from reaction of dichlorone with thiophenol; and (C) 2,3-di-*n*-octylthiopyran-1,4-naphthoquinone derived from reaction of dichlorone with *n*-octylmercaptan. KBr pellet, 1.0 mg., 300 mg. KBr.

The reaction can be written as shown above for 2-nitrothiophenol.

**Reaction of dichlorone with glutathione.** Glutathione, 92 mg. in 2.5 ml. of water, was neutralized with NaOH and mixed with 34 mg. of dichlorone in 50 ml. of absolute ethanol with constant stirring. Five ml. of 2 per cent aqueous sodium acetate solution were added to prevent a large drop in pH. After incubation for 1 hour the solution was poured into 9 volumes of acetone and a reddish precipitate was obtained. The material was soluble in water but not in acetone or absolute ethanol. It did not crystallize upon evaporation of the water from an aqueous solution or upon mixing with acetone or ethanol. Elemental analyses did not agree with values calcu-

lated for the disubstituted or monosubstituted derivative but were intermediate. This suggested that the precipitate was a mixture of mono- and disubstituted quinone.

The purity of the precipitate was then tested by paper chromatography. A sample was spotted on Whatman No. 1 chromatography paper and run ascending in butanol-acetic acid-water (40:10:50 v/v), upper phase. Three spots separated. The major fraction was bright orange and had an  $R_f$  value of 0.26. A reddish-brown spot moved to  $R_f$  0.38 and a small yellow component was located at  $R_f$  0.52. It is assumed from this and from the elemental analyses that the precipitate was a mixture of mono- and disubstituted quinone with a trace of unreacted dichlone. Infrared patterns showed little that was distinctive about the compounds, perhaps because of the size of the molecules and the variety of groups present. The absorption spectrum at visible and ultraviolet wavelengths showed no characteristic features except absorption in the vicinity of  $450\text{ m}\mu$  in the visible and  $270\text{ m}\mu$  in the ultraviolet, which was exhibited by all of the thiol derivatives.

*Reaction of dichlone with CoA.* Dichlone, 6.75 mg. in 25 ml. of ethanol, was mixed with an aqueous solution of 30 mg. of CoA and 25 mg. of sodium acetate. The mixture was stirred for one hour and then poured into 9 volumes of acetone. A brown precipitate was obtained which could not be crystallized. Evaporation of the supernatant solution resulted in a residue of a considerable amount of dichlone, which indicates that the reaction was not complete. Because of the small amount of CoA available, further experiments were not carried out. The product exhibited an absorption maximum in the vicinity of  $450\text{ m}\mu$ , typical of other thiol derivatives of dichlone. It is assumed, therefore, that the reaction product(s) were analogous to those derived from reaction of glutathione with dichlone.

#### REACTION OF CAPTAN AND THIOPHOSGENE WITH THIOLS

*Reaction of captan with 4-nitrothiophenol.* 4-Nitrothiophenol was used to determine the number of moles of thiol capable of reacting with each mole of captan, and the number of chlorine atoms liberated in the reaction. The data in Table I show that 4 moles of 4-nitrothiophenol reacted with each mole of captan present. Three atoms of chlorine were released. The 4:1 ratio was independent of the initial ratio of thiol to captan. This shows that the reaction was not a simple stoichiometric substitution of chlorine, because only 3 chlorine atoms are present in the captan molecule.

In a subsequent experiment, the products of the reaction were isolated and characterized by elemental analyses. 4-Nitrothiophenol, 400 mg. in 20 ml. of acetone, was mixed with 190 mg. of captan in 20 ml. of acetone at room temperature. Sodium acetate, 700 mg. in 10 ml. of water, was added as a buffer. A crystalline precipitate formed almost immediately.



Without further purification the crystals melted at  $181-182^{\circ}\text{C}$ . This was close to the melting point of  $183-184^{\circ}\text{C}$ . for authentic bis(4-nitrophenyl) disulfide. The product reacted with colorless thiols at pH 8.0 to produce the intensely yellow 4-nitrothiophenate, as does authentic bis(4-nitrophenyl) disulfide (3), and the infrared patterns of the two compounds were identical (Fig. 3 A). This proves that the product was bis(4-nitrophenyl) disulfide.

The mother liquor was warmed and water was added dropwise until the solution was slightly cloudy. It was then allowed to cool and a second product crystallized as yellow needles, m.p.  $128-130^{\circ}\text{C}$ . The possibility that the compound might be bis(4-nitrophenyl) sulfide was eliminated on the basis of melting point, since an authentic sample of the sulfide melted

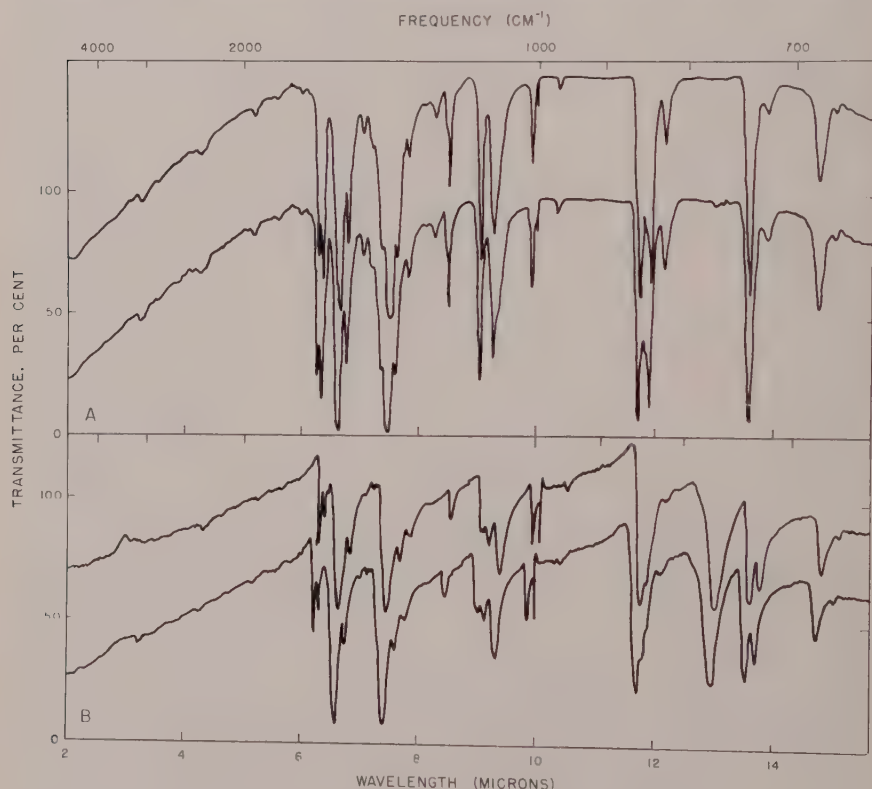


FIGURE 3. Infrared absorption spectra of: (A) authentic bis(4-nitrophenyl) disulfide (upper curve) and the disulfide formed in the reaction of captan with 4-nitrothiophenol (lower curve) (KBr pellets, 1.3 mg./300 mg. KBr), and (B) bis(4-nitrophenyl) trithiocarbonate formed in the reaction of 4-nitrothiophenol with captan (upper curve) and with thiophosgene (lower curve) (KBr pellets, 1.0 mg./300 mg. KBr).

at 165–167° C. It was then suspected that the compound might be bis(4-nitrophenyl) trithiocarbonate. This was confirmed by elemental analyses.

*Anal.* Calcd. for the trithiocarbonate,  $C_{13}H_8N_2O_4S_3$ : C, 44.4; H, 2.3; N, 7.9; O, 18.2; S, 27.2. Found: C, 42.1; H, 2.4; N, 9.6; O, 21.5; S, 25.4.

The aqueous solution from which the trithiocarbonate was crystallized was reduced in volume in a stream of air and white plate-like crystals were obtained, m.p. 134–135° C. This was close to the m.p. of 133–134° C. reported by Lukens for tetrahydrophthalimide (7).

*Reaction of thiophosgene with 4-nitrothiophenol.* Since thiophosgene has been postulated to be an intermediate in the reaction of captan with thiols (7, 8), an experiment was carried out to compare the products of the reaction of thiophosgene and 4-nitrothiophenol with that obtained with captan. Thiophosgene, 0.095 ml., and 390 mg. of 4-nitrothiophenol were mixed in 30 ml. of acetone. Sodium acetate, 500 mg. in 5 ml. of water, was added as a partial buffer. The yellow 4-nitrothiophenate ion disappeared almost immediately, indicating an extremely rapid reaction. The solution was heated and water was added dropwise until the solution was slightly cloudy. Upon cooling, yellow needle-like crystals formed, melting at 130–132° C. This was close to the melting point of 128–130° C. obtained for bis(4-nitrophenyl) trithiocarbonate from the reaction of captan with 4-nitrothiophenol.

*Anal.* Calcd. for the trithiocarbonate,  $C_{13}H_8N_2O_4S_3$ : C, 44.4; H, 2.3; N, 7.9; O, 18.2; S, 27.2. Found: C, 45.8; H, 2.9; N, 8.5; O, 16.9; S, 27.1.

The data indicate, therefore, that thiophosgene and captan give rise to an identical product upon reacting with 4-nitrothiophenol, although captan also yields a disulfide which is not formed in the thiophosgene reaction. This was confirmed by absorption spectra in the ultraviolet and infrared regions of the spectrum (Figs. 3 B and 4 A). In all cases the curves for the derivatives were identical.

*Rates of reaction of captan and thiophosgene with 4-nitrothiophenol.* In order to compare the rates of reaction of captan and thiophosgene with 4-nitrothiophenol, it was necessary to lower the pH sufficiently to retard the reaction. It was found that the time required for the half reaction ( $t_{50}$ ) of captan was sufficiently long for convenient measurements when the solutions were buffered between pH 1.2 and 2.2. 4-Nitrothiophenol was mixed with captan in the ratio 4:1 in 1:1 buffer—acetone solutions. The time required for a 50 per cent decrease of the color density of the solution at each pH unit from 1.2 to 2.2 was then determined. Density measurements were made with a Beckman Model DU spectrophotometer at a wavelength of 412 m $\mu$ . The results are given in Figure 5. The data show that the reaction was markedly retarded as the pH was lowered below 2.2. The color intensity of the controls prepared without captan also di-

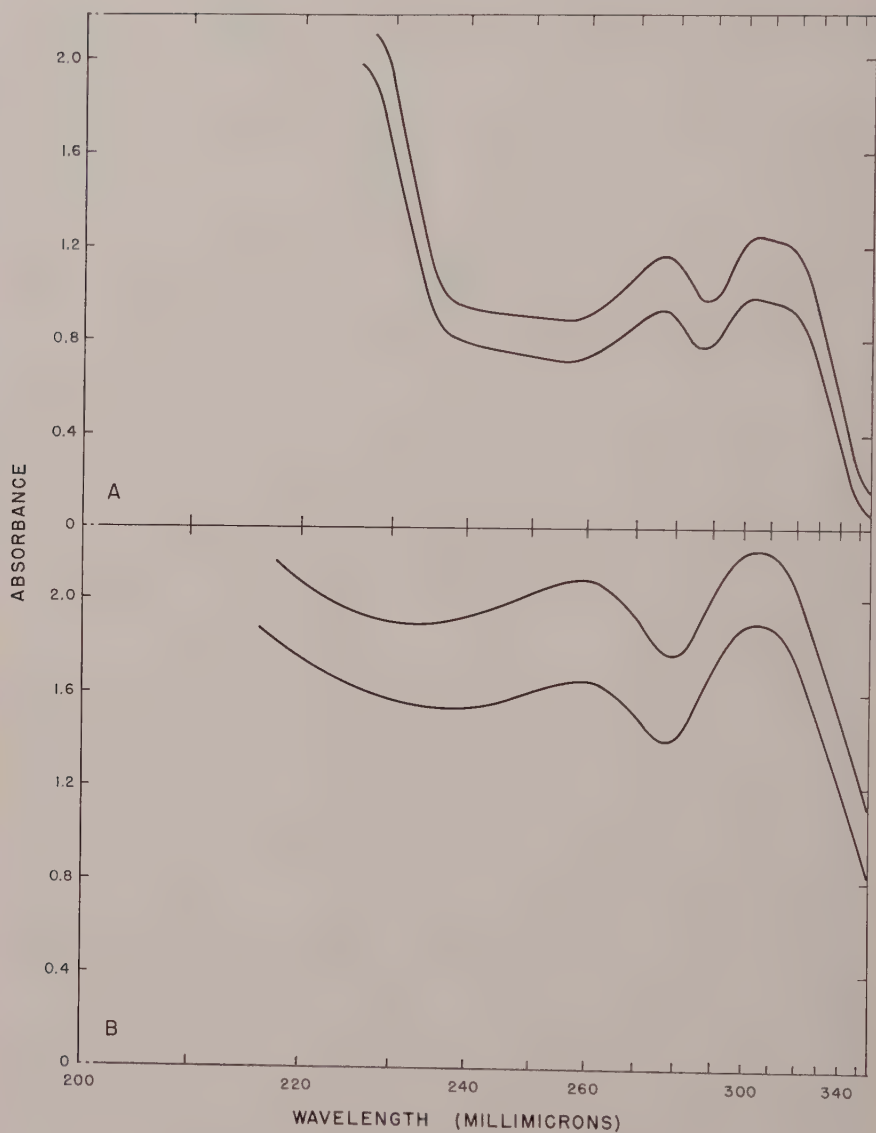
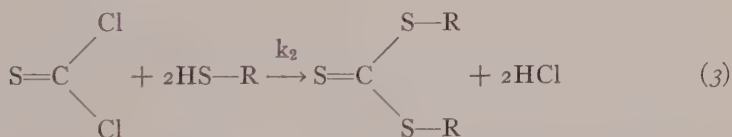
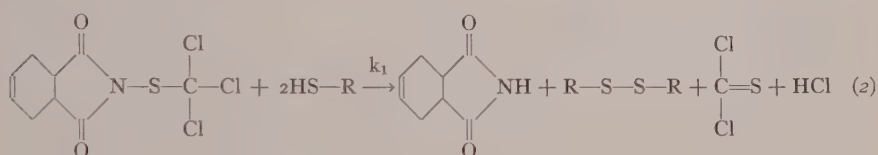


FIGURE 4. Ultraviolet absorption spectra of: (A) bis(phenyl) trithiocarbonate formed in the reaction of thiophenol with captan (upper curve) and with thiophosgene (lower curve) (concentration, 30  $\mu\text{g./ml.}$ ) and (B) bis(4-nitrophenyl) trithiocarbonate formed in the reaction of 4-nitrothiophenol with captan (upper curve) and with thiophosgene (lower curve) (concentration, 35  $\mu\text{g./ml.}$ ). Solvent, absolute ethanol.

minished due to decreased ionization of 4-nitrothiophenol. This suggests that the rate of reaction with captan was dependent upon the concentration of ionized 4-nitrothiophenol.

An identical experimental procedure was used with thiophosgene in place of captan. However, the reaction with thiophosgene was complete in less than 15 seconds even at pH 1.2. The experiments show, therefore, that the velocity of the reaction of 4-nitrothiophenol with thiophosgene was many times greater than with captan. Thus, the rate-limiting step in the over-all reaction with captan was the initial step involving the intact captan molecule. The immediate reaction of thiophosgene, upon formation from captan, accounts for the fact that no thiophosgene *per se* was detectable in the reaction system. This is in contrast to the evolution of thiophosgene vapor reported by Lukens and Sisler (8) upon reacting cysteine with captan and in the reaction of glutathione with captan, which is reported in a subsequent section of this paper.

The over-all reaction between captan and 4-nitrothiophenol can be visualized as occurring in two steps, as postulated previously for the dialkyldithiocarbamates by Lukens (7):



In these reactions,  $k_1$  is the rate constant for the initial step in which the disulfide, tetrahydrophthalimide, thiophosgene, and HCl are formed. With 4-nitrothiophenol,  $k_1$  was much smaller than  $k_2$ , the rate constant for the substitution of thiophosgene in step 2.

*Reaction of captan with thiophenol:* Thiophenol, 1.48 g., was mixed with 1.0 g. of captan in 90 ml. of ethanol and 2.5 g. of sodium acetate in 10 ml. of water were added. After about an hour, an equal volume of water was added and the mixture was extracted several times with ethyl ether. The ether fraction was dried in a stream of air. The residue was dissolved in acetone and cooled to  $-10^\circ\text{C}$ . in a dry ice bath. Water was added dropwise with stirring until a white crystalline precipitate formed, m.p.  $59-61^\circ\text{C}$ . The melting point was close to that of authentic phenyl disulfide ( $61-62^\circ\text{C}$ .).

After removal of the white crystals by filtration, more water was added



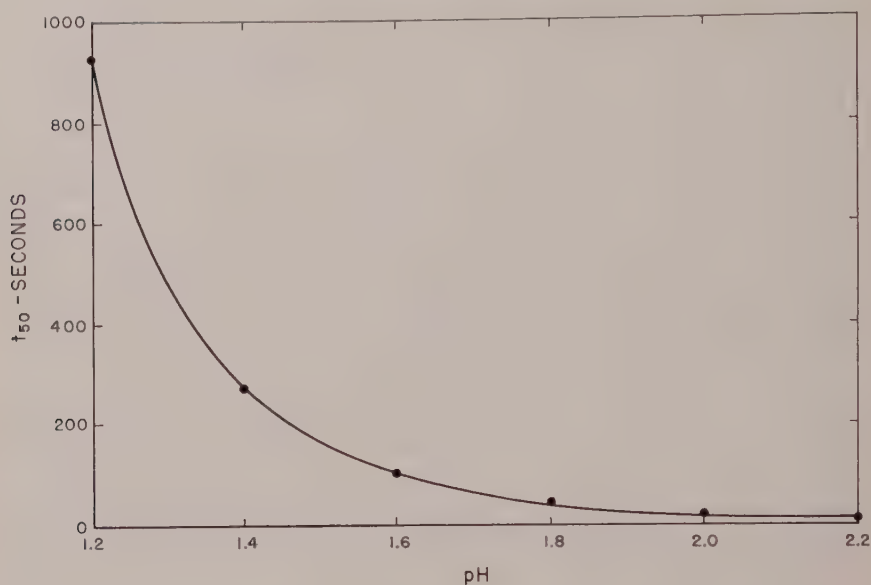


FIGURE 5. Relationship between pH of the reaction medium and the time for 50 per cent reaction ( $t_{50}$ ) of 4-nitrothiophenol with captan in 1:1 acetone—aqueous buffered solution.

to the filtrate until the solution was slightly cloudy. Upon standing in a dry ice bath, yellow crystals formed, m.p.  $94-95^{\circ}\text{C}$ . This fraction was considered to be analogous to the trithiocarbonate formed from 4-nitrothiophenol. This was confirmed by elemental analyses.

*Anal.* Calcd. for the trithiocarbonate,  $\text{C}_{13}\text{H}_{10}\text{S}_3$ : C, 59.6; H, 3.8; S, 36.6. Found: C, 60.0; H, 3.8; S, 35.6.

*Reaction of thiophosgene with thiophenol.* Thiophenol, 0.265 ml., and 0.1 ml. of thiophosgene were combined in 9 ml. of acetone, and 250 mg. of sodium acetate in 5 ml. of water were added. After a few minutes the solution was cooled in a dry ice bath and water was added until the solution was slightly cloudy. Upon standing, yellow crystals separated, m.p.  $92-94^{\circ}\text{C}$ . The melting point was close to that of the trithiocarbonate derived from the reaction with captan reported above ( $94-95^{\circ}\text{C}$ .).

*Anal.* Calcd. for the trithiocarbonate,  $\text{C}_{13}\text{H}_{10}\text{S}_3$ : C, 59.6; H, 3.8; S, 36.6. Found: C, 59.9; H, 4.1; S, 35.7.

The analyses suggest that the product was the trithiocarbonate identical to that formed in the reaction with captan. This was confirmed by absorption spectra. Absorption of the derivatives was identical at ultra-violet and infrared wavelengths (Figs. 4 B and 6 A).

*Reaction of captan with glutathione.* A number of tests were carried out with glutathione because it was found to react with captan in a proportion

different from that of 4-nitrothiophenol and thiophenol. Unlike the 4:1 molar ratio for the 4-nitrothiophenol-captan reaction, the ratio with glutathione averaged about 2.5:1 in dilute mixtures. In addition, the odor of thiophosgene was distinct, in contrast to the lack of any trace of thiophosgene in the reaction between captan and the thiophenols. The evolution of thiophosgene shows that glutathione reacted more rapidly with intact captan than with the thiophosgene that was generated, for had the converse been true, free thiophosgene would not have been detectable.

An experiment was carried out to recover the thiophosgene formed during the reaction. The apparatus consisted of a reaction flask equipped with a condenser. The condenser passed through a jacket packed with dry ice and emptied into a filter flask connected to the laboratory vacuum line. The filter flask was charged with an acetone solution of 4-nitrothiophenol buffered at about pH 7.0 with sodium acetate. The flask was kept under vacuum and immersed in dry ice during the reaction.

The reaction mixture consisted of 3.3 millimoles of neutralized glutathione and 3.3 millimoles of captan. The glutathione was dissolved in 60 ml. of water and the captan in 60 ml. of acetone. The solutions were mixed and allowed to react for 30 minutes with constant stirring. Periodic checks for sulfhydryl groups showed that all of the glutathione had reacted within the 30-minute period. At the end of the reaction period the reaction mixture was warmed gently under vacuum to about 25° C. to distill the acetone and thiophosgene over into the trapping flask containing 4-nitrothiophenol. When the temperature began to rise, distillation was halted and the apparatus was disassembled.

The trapping solution was treated with 50 ml. of 10 per cent aqueous sodium acetate to convert the excess 4-nitrothiophenol to the sodium salt. The solution was then extracted several times with ethyl ether to dissolve any bis(4-nitrophenyl) trithiocarbonate that had formed. The ether extract was washed several times with aqueous sodium acetate solution to remove traces of 4-nitrothiophenol. The ether was then removed in a stream of air and the residue was taken up in a small amount of acetone. The acetone solution was warmed and water was added dropwise until the solution was slightly cloudy. Upon cooling and standing, 29 mg. of yellow needle-like crystals separated. The crystals melted at 127–129° C. which was close to the m.p. of 130–132° C. for bis(4-nitrophenyl) trithiocarbonate obtained from the reaction between thiophosgene and 4-nitrothiophenol. This showed that only 0.0825 millimole of a theoretical 3.3 millimoles of thiophosgene was trapped.

The mixture remaining in the reaction flask was transferred to a separatory funnel and extracted with several portions of ethyl acetate to dissolve the residual captan. The solvent was removed in a stream of air and 650 mg. of residue melting at 168–170° C. was recovered. Thus, only 350

of the original 1000 mg. of captan reacted, whereas all of the glutathione had reacted. Therefore, the glutathione—captan molar ratio was 2.8:1. Since only 1.17 millimoles of captan reacted, the 0.0825 millimole of thiophosgene trapped was actually 7 per cent of the theoretical amount.

The products remaining in the aqueous phase after removal of the residual captan were passed into a  $2 \times 8$  cm. column of formate form Dowex 50-X8 resin, 200 to 400 mesh. The effluent liquid was evaporated in a stream of air and yielded 109 mg. of tetrahydrophthalimide, m.p.  $125-127^\circ \text{C}$ .

The glutathione product, which was retained by the column, was eluted with 1*N* aqueous  $\text{NH}_4\text{OH}$  and then dried in a stream of air. The residue weighed 1.043 g., which was about 2 per cent more than the theoretical amount of glutathione. The product could not be crystallized.

In another experiment the reaction product was separated by ascend-

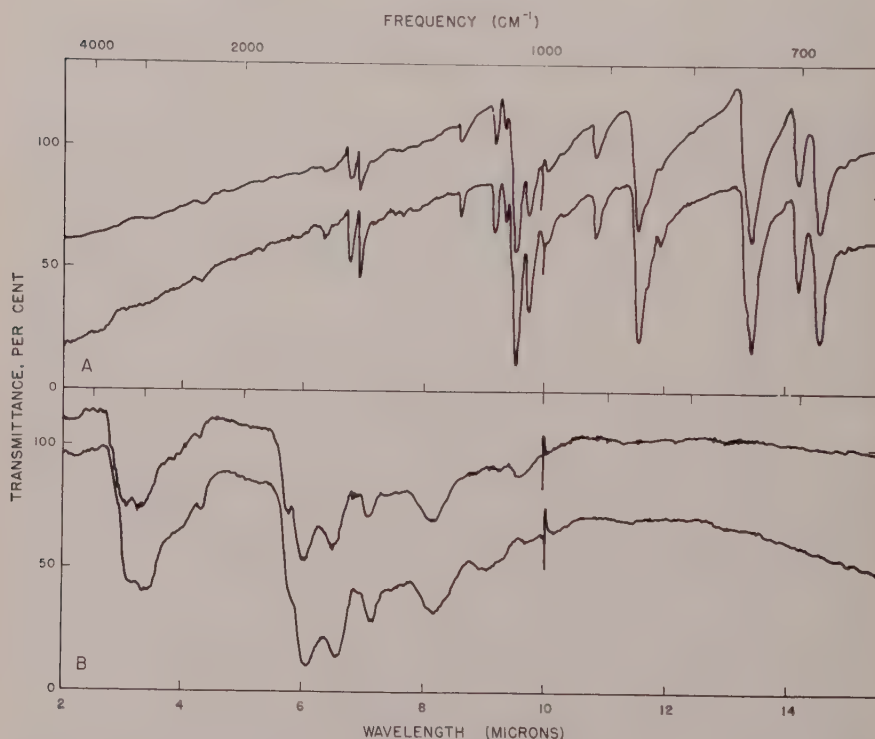


FIGURE 6. Infrared absorption spectra of: (A) bis(phenyl) trithiocarbonate formed in the reaction of thiophenol with captan (upper curve) and with thiophosgene (lower curve) (KBr pellets, 1.3 mg./300 mg. KBr), and (B) authentic oxidized glutathione (upper curve) and the product formed in the reaction of glutathione with captan (lower curve) (KBr pellets, 1.0 mg./200 mg. KBr).

ing chromatography on Whatman No. 1 paper with butyl alcohol-formic acid-water (14:3:3 v/v) as the solvent. Glutathione and oxidized glutathione were run simultaneously for reference. Triple development was used, the papers being dried between each development. The spots were then made visible by treatment of the papers with 1 per cent ninhydrin in ethanol (15). The reaction product yielded one spot which corresponded to oxidized glutathione.

Subsequently, the infrared absorption spectrum of the compound was determined and compared with that of authentic oxidized glutathione. The curves are given in Figure 6 B. The infrared patterns, like the paper chromatograms, revealed no essential difference between the product from the glutathione-captan reaction and oxidized glutathione. No indication of a trithiocarbonate was obtained. However, the possibility that the trithiocarbonate was formed cannot be unequivocally eliminated because it might have decomposed in such a manner as to yield oxidized glutathione and some byproduct such as thiocarbonate. The fact that only about 7 per cent of the theoretical amount of thiophosgene was recovered in the trapping experiment above suggests that there was a reaction in which thiophosgene was decomposed. In any event, the trithiocarbonate was apparently absent or present in undetectably small amounts in the final product. Had it been present in appreciable amounts, the infrared curves should have shown one or more peaks of reasonable magnitude for the C=S group in the frequency range from 1200 to 1050  $\text{cm}^{-1}$ , as shown by the other trithiocarbonates in Figures 3 B and 6 A.

#### DISCUSSION

Previous reports on the reaction of dichlone with thiols have assumed that the rate of substitution of the second chlorine atom on the ring would be greatly diminished or completely hindered by substitution of the first. However, the foregoing experiments show that simple, low molecular weight thiols react rapidly at the site of the second as well as the first chlorine atom, particularly in the presence of salts of weak acids which maintain the pH of the reaction medium at physiological values. However, more complex, higher molecular weight thiols, such as glutathione and CoA, yield a mixture of mono- and disubstituted products. This suggests that substitution of the second chlorine atom is somewhat impeded, possibly as a result of steric hindrance and the relatively high  $\text{pK}_a$  of glutathione and CoA.

Apparently dichlone will react with any thiol having accessible sulfhydryl groups, whether the reaction is carried out under controlled conditions *in vitro* or whether the reactants are components of living cells. This is supported by a considerable amount of biochemical data. It was shown previously that enzymes with functional sulfhydryl or amino groups are



inactivated by dichlone (9, 10, 11, 12). Later studies on intact spores of *Neurospora sitophila* (Mont.) Shear & Dodge showed that toxicity of dichlone is due to concomitant inhibition of phosphorylation, certain dehydrogenases, carboxylases, and CoA (12). Each of these is known to be sulfhydryl-dependent.

A further consequence of indiscriminate reaction with sulfhydryl compounds is that much of the dichlone that enters the cells will react with excess glutathione, cysteine, and perhaps other substances in the free amino acid and peptide pools. Thus extraneous thiols, and perhaps amino acids and proteins, probably act as protective agents by virtue of the same kinds of chemical reactions involved in toxicity. The pH of the cytoplasm probably also plays a role in toxicity, because the rate of reaction of dichlone decreases with increasing acidity.

The chemistry of the reactions of thiols with captan is considerably more complicated than with dichlone. Thiols such as 4-nitrothiophenol and thiophenol are converted to disulfides and stable trithiocarbonates. Captan is partially decomposed to tetrahydrophthalimide and hydrochloric acid and contributes the methyl carbon and a sulfur atom to the trithiocarbonate. Some thiols, such as the dialkyldithiocarbamates studied by Lukens (7), apparently form unstable trithiocarbonates which break down to yield carbon disulfide and a tetraalkylmonosulfide. Still others, such as cysteine, react with captan to yield ring compounds (8) by virtue of the presence of an amino group which takes part in ring closure. It is apparent, therefore, that a variety of derivatives can result from captan interaction with different kinds of thiols.

Formation of thiophosgene as an intermediate in the reactions of captan with thiols now seems well established. It was detected by Lukens and Sisler (8) in the reaction between captan and cysteine and was postulated as an intermediate in the reaction of captan with dialkyldithiocarbamates (7), although its presence was not demonstrated directly. The present studies show that it reacts too rapidly to be detectable in the presence of certain thiols, but in the reaction of glutathione with captan it is easily detectable by organoleptic test and can be distilled from the reaction mixture and trapped by the formation of bis(4-nitrophenyl) trithiocarbonate from 4-nitrothiophenol.

The fact that free thiophosgene is detectable in certain reactions is strong evidence, of course, that it reacts more slowly than it is formed in these particular reactions. This difference in rate of reaction of thiophosgene with different thiols accounts for the difference in thiol—captan ratio found in the foregoing experiments. With 4-nitrothiophenol and thiophenol free thiophosgene was never detectable and the thiol—captan ratio for the reaction was always 4:1 regardless of the molecular ratio in which they were mixed. This is undoubtedly due to the fact that thiophosgene reacts more rapidly with available thiol than does intact captan. In the case of

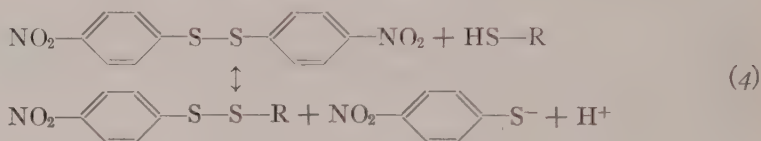
glutathione, however, the reaction ratio was found to be approximately 2.5 to 3:1 and free thiophosgene was recoverable. The fact that thiophosgene was recoverable may be taken as proof that it reacted more slowly with glutathione than did intact captan. The basis of the differential in rates with different thiols has not been studied specifically, but it seems likely that factors such as steric hindrance and  $pK_a$  differences play a role in determining reaction rates in some instances. In addition, lyophilicity might also have an influence. For example, glutathione, being hydrophilic, is probably surrounded by water dipoles in aqueous mixtures of solvents whereas thiophosgene would be surrounded by molecules of the organic solvent. These lyophile layers might act as barriers and retard the rate of effective collisions between the reactants. Since the thiophenols and thiophosgene possess mutual affinities for the organic solvents, no solvent barrier would exist between them.

Thus in the reaction between cellular thiols and captan there is probably a competition for available sulphydryl groups between intact captan and the thiophosgene formed from it. This has a bearing on the theory that thiophosgene is the ultimate toxicant in the action of captan on fungi (7, 8). The foregoing data suggest that captan would react more rapidly than thiophosgene with the hydrophilic thiols found in cells. This tends to cast doubt on the importance of thiophosgene in the mechanism of action of captan because spores of *Neurospora sitophila* were shown to contain only about 2 mg. of thiols, as glutathione equivalents (14), whereas 8 mg. of captan are required to inhibit germination (13). Thus there is a rather large excess of captan absorbed before germination is completely inhibited, which, in view of the more rapid reaction of captan, should favor the intact captan-thiol reaction.

In the trapping experiment described above, only a small portion of the theoretical amount of thiophosgene was recovered from the reaction between glutathione and captan. All of the original 3.3 millimoles of glutathione but only 1.17 millimoles of captan reacted. Since this amount of captan accounts for reaction of only 2.34 millimoles of glutathione, it must be assumed that 0.96 millimole of glutathione reacted with thiophosgene. This would have required 0.48 of the theoretical 1.17 millimoles of thiophosgene that should have formed, leaving approximately 0.6 millimole of thiophosgene. Of this only 0.0825 millimole was recovered by trapping. The fate of the remainder is unknown, but hydrolysis is probably too slow to account for its disappearance (1). It is possible that losses in the trapping procedure were high enough to account for the discrepancy, but it seems more probable that the thiophosgene reacted with amino groups of glutathione or that a glutathione trithiocarbonate was formed but decomposed spontaneously to oxidized glutathione and thiocarbonate. Further studies are required to resolve these questions.

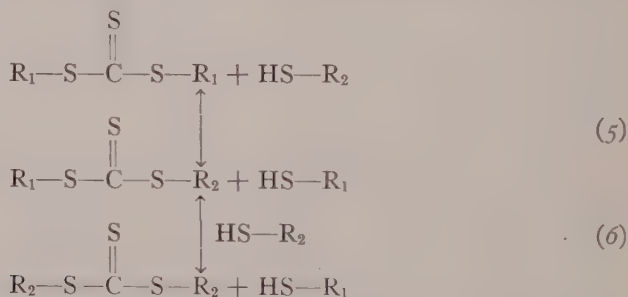
It would seem worthwhile at this point to consider the nature of the

trithiocarbonate and other products that are formed in the reaction between thiophosgene and various thiols. It was shown previously (11) that the sulfhydryl groups of CoA and glutathione disappeared in the presence of captan. However, both compounds were quantitatively restored to the thiol form by addition of excess thiophenol. Reversal of the reaction was postulated to occur as a result of an exchange reaction. The exchange reaction is clearly exemplified by Ellman's reaction which is used as a specific assay for sulfhydryl compounds (3). This reaction involves exchange of an intensely colored 4-nitrothiophenolate ion from bis(4-nitrophenyl) disulfide for colorless thiols in slightly alkaline solution:



Fava *et al.* (4) have recently studied the kinetics of thiol-disulfide exchanges and their work indicates that the reaction takes place readily and continues until an equilibrium is reached.

Trithiocarbonates also undergo the same kinds of exchanges.<sup>3</sup> Bis(4-nitrophenyl) trithiocarbonate can be substituted for bis(4-nitrophenyl) disulfide in Ellman's assay procedure. The reaction can be generalized as follows:



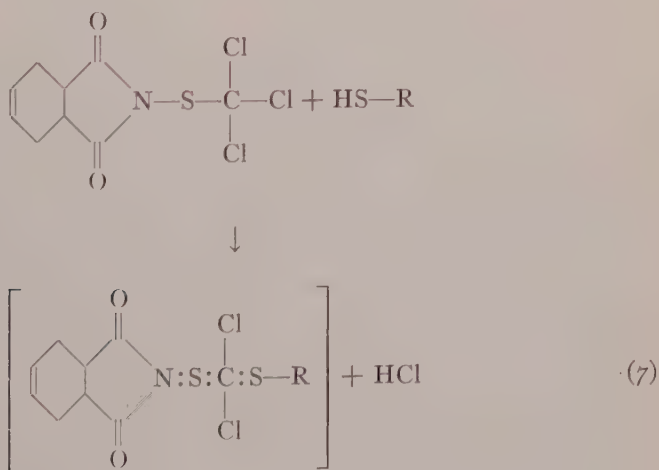
Since the exchange is an equilibrium reaction, a large excess of the thiol will favor more nearly complete exchange.

These reactions account for the restoration of CoA and glutathione to the free sulfhydryl forms, as reported previously (11). Obviously, since the trithiocarbonate from reaction of thiols with thiophosgene and the disulfide from reaction with captan are equally susceptible to exchange and liberation of the original thiol, the two toxicants would be equivalent with respect to *in vivo* effects if thiols were the only components with which they reacted since the toxicity of both could be reversed. Evidence was pre-

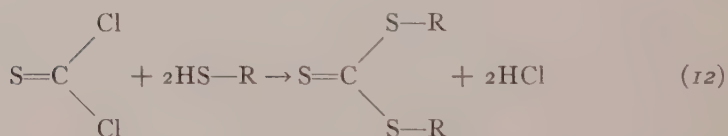
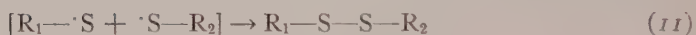
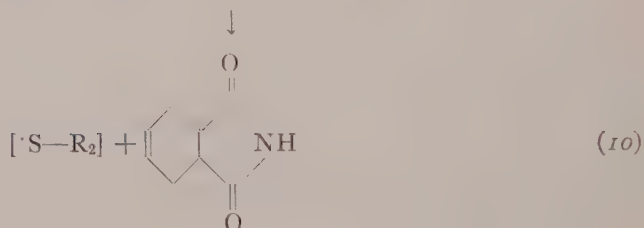
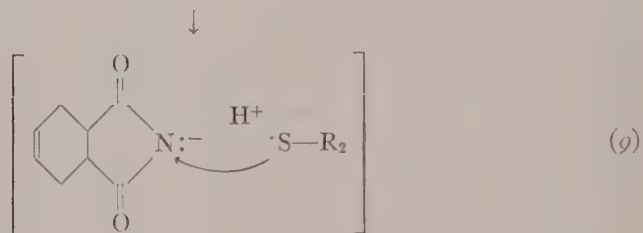
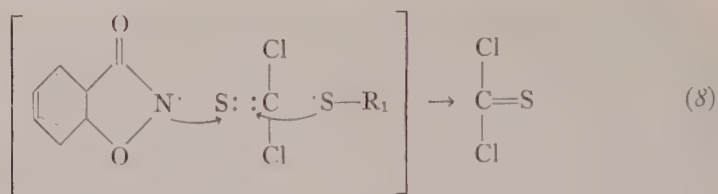
<sup>3</sup> Unpublished data.

sented previously (13) that the effects of captan on respiration and germination of *N. sitophila* spores are partially or wholly reversed spontaneously, depending upon the initial dose of captan. It was shown that 5 mg. of captan g. of spores (dry wt.) strongly inhibited  $O_2$  uptake and  $CO_2$  release. Recovery of viability of the spores was also demonstrated. Spores that had absorbed 6 mg. of captan g. of spores were inhibited 60 per cent 8 hours after treatment whereas only 20 per cent were inhibited after 12 hours. It should be pointed out, however, that the tendency to recover was less marked when the dose of captan was increased. This was probably due to the presence of excess captan which reacted with all thiols capable of reversing the reaction. In any event, autoreversibility of inhibition suggests that captan reacted in such a way as to form disulfides or trithiocarbonates which decomposed to liberate functional thiols. In view of the relatively slow rate of reaction of thiophosgene with thiols such as glutathione, and the large excess of intact captan over thiophosgene, it seems most probable that the reversible reaction was formation of the disulfide rather than of the trithiocarbonate.

Another point of considerable interest is the chemical mechanism of the captan-thiol reaction. Formation of a disulfide shows that the net result of the reaction is the loss of an electron by each of 2 thiol molecules. The following representation of hypothetical electron shifts could account for the products obtained:

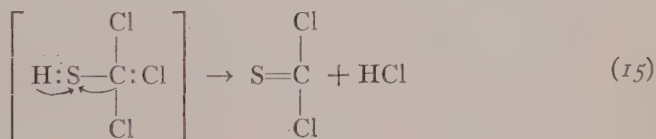
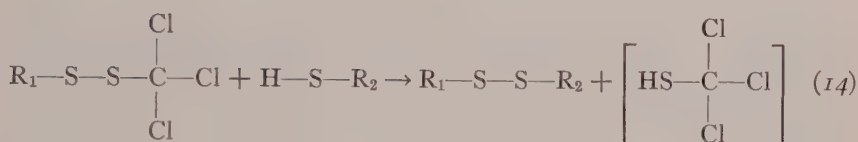
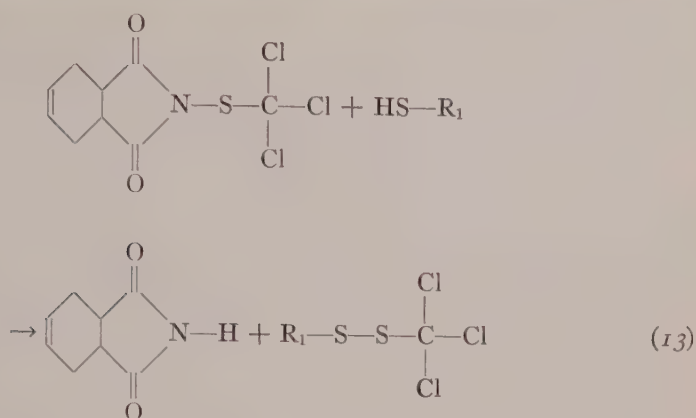






In this scheme the first step in the reaction is the substitution of one chlorine atom on the perchloromethyl carbon of captan. This results in an unstable intermediate in which one electron from the N—S bond and one from the C—S bond shift orbits and pair to form the C=S bond. This results in a tetrahydrophthalimide and a thiol free radical. The tetrahydrophthalimide free radical captures an electron from a second thiol molecule, creating a second thiol free radical, which combines with the first to form a stable disulfide. The final step is a straightforward substitution of thiophosgene, which yields the trithiocarbonate.

This mechanism, of course, needs confirmation by direct evidence of free radicals, because an alternative mechanism is possible; e.g., a polar process involving the attack of the thiol anion on the N—S bond of captan. This bond is somewhat analogous to the S—S bond of disulfides, which can take part in exchange reactions. The exchange can be visualized as follows:



A similar exchange mechanism was suggested by Lukens and Sisler (8) but the present state of knowledge does not permit a choice between the two possible mechanisms, since both would yield the same products.

The fact that glutathione reacted more rapidly with intact captan than with thiophosgene, whereas the relative rate is reversed in the case of 4-nitrothiophenol, might be taken as a hint that the exchange mechanism is the predominant one. If substitution of captan were the initial reaction, the relative rates of substitution of captan and thiophosgene by different thiols should always be the same, e.g., substitution of thiophosgene is greater than substitution of captan, since the controlling factors would always exert control in the same direction regardless of the thiol employed. However, if two intrinsically different kinds of reactions were involved, the order of reaction rates might be changed because of the nature of the thiol and its propensity to participate in the different kinds of reactions. Although this kind of argument is not without theoretical foundation, the question cannot be resolved without further study.

It is concluded from the present study that simple thiols will react with dichlone to form a disubstituted quinone. The reaction is a substitution

reaction involving the chlorine sites on the ring. More complex thiols, such as those normally found in the cells of living organisms, apparently do not react so completely, but yield mixtures of mono- and disubstituted quinones. The reaction is generally irreversible.

The reaction of thiols with captan is more complicated than that with dichlone. Captan is partially decomposed in the reaction and part of the thiol is oxidized to the corresponding disulfide and a portion is converted to the corresponding trithiocarbonate, apparently by a substitution reaction involving thiophosgene, which is formed as a result of the initial reaction between intact captan and the thiol. Some thiols such as thiophenols give rise to stable trithiocarbonates, whereas others such as dialkyldithiocarbamates yield unstable trithiocarbonates which decompose to yield  $\text{CS}_2$  and the corresponding monosulfide (7). [Glutathione, and probably other thiols of the kinds found in living cells, show less propensity to react with thiophosgene than with intact captan, so that thiophosgene vapor may be evolved. It is suggested that the thiophosgene may react in cells with amino compounds in view of the thiol : captan ratio in cells at the lethal dose of captan, which heavily favors direct reaction between the thiols and intact captan.]

The reaction of thiols with captan and/or thiophosgene is reversible, since the disulfides and trithiocarbonates readily undergo exchange reactions with free thiols to liberate the original thiol. Disulfides, moreover, may be reduced without anion exchange under appropriate conditions. These conclusions are in agreement with the biochemical and chemical data available to date and appear to substantiate the previous concepts of the mechanism of action of dichlone and captan.

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